THE UNIVERSITY OF ALBERTA

EXPERIMENTAL CANCER CHEMOTHERAPY WITH CYTOTOXIC NUCLEOSIDES AND NUCLEOSIDE TRANSPORT INHIBITORS

by

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DEDICATION

TO

Kwasi Boakye who made all this possible.

AND

Adwoa Boatemaa who did not live to see any of this.

ABSTRACT

Nitrobenzylthioinosine (NBMPR) is a potent, specific inhibitor of nucleoside transport (NT) in animal cells of several types. Combinations of NBMPR with potentially lethal doses of cytotoxic nucleosides have led to substantial kill of tumour cells in mice and, as well, have protected the host mice from the toxicity of the former, evidently by preventing the access of the toxicant to dose-limiting tissues. The present study has extended these findings by studying various cytotoxic nucleoside-NT inhibitor combinations and different routes of inhibitor administration. The host-protecting capacity of the inhibitors differed widely and did not correlate with their *in vitro* NT inhibiting activities. The rank order of their potencies also varied with route of administration, suggesting that these differences were attributable to pharmacokinetic properties. Several of the inhibitors yielded significant responses in tumour-bearing mice, on intraperitoneal or subcutaneous co-administration with toxic nucleosides, notably tubercidin or nebularine.

In attempting to adapt the "host-protection" tactic to the therapy of human neoplastic disease, we explored nucleoside permeation characteristics in a human ovarian carcinoma cell line (HOC-7). Processes contributing to nucleoside permeation in HOC-7 cells were (i) a minor (30-35%) facilitated diffusion component, and (ii) a major (65-70%) transporter-independent component of influx, that was not temperature-dependent and was of low sentivity to NBMPR. The latter pathway lacked enantiomeric selectivity in that it permited the influx of β-L-adenosine, the "mirror-image" isomer of β-D-adenosine. Also, fluxes of physiological nucleosides via this pathway were independent of lipophilicity of the nucleosides. Transporter-independent nucleoside permeation in HOC-7 cells was therefore attributed to a channel-like or pore-like route. HEY and HOC-1, two other cultured lines of human ovarian carcinoma cells, also expressed the channel-like nucleoside permeation pathway, which was not expressed by SK-OV-3, another human ovarian carcinoma line.

NBMPR failed to protect HEY and HOC-7 cells against the antiproliferative effects of cytotoxic nucleosides in culture, indicating the therapeutic activity of nucleoside analogue-NT inhibitor combinations in chemotherapy of these neoplasms.

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ABBREVIATIONS

Ado	β-D-adenosine
L-Ado	β-L-adenosine
ara-C	9-β-D-arabinofuranosylcytosine
B_{max}	maximum number of ligand binding sites per cell as determined from equilibrium binding data by mass law analysis
dAdo	2'-deoxyadenosine
dilazep	3,4,5-trimethoxybenzoic acid diester with tetrahydro-1H-1,4-diazepine-1,4(5H) -dipropanol
dipyridamole	2,2',2'',2'''-(4,8-dipiperidinopyrimido[5,4d] pyridine-2,6-diyldinitrilo)tetraethanol -2,6-diyldinitrilo)tetraethanol
dThd	thymidine
FaraA	9-β-D-arabinofuranosyl-2-fluoroadenine
Guo	guanosine
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
hexobendine	3,4,5-trimethoxybenzoic acid 1,2-ethanediylbis[(methylimino)3,1-propanediyl] ester
HPLC	high-performance liquid chromatography
IC_{50}	concentration at which 50% inhibition occurs
Ino	inosine
ip	intraperitoneal
K_D	dissociation constant
K_i	inhibition constant
K_m	substrate concentration for half-maximal unidirectional flux(analogous to the Michaelis-Menten constant)
LD_{50}	drug dose required to kill 50% of treated animals
α-MEM	minimum essential medium for monolayer cultures
NBdAdo-P	N^6-(4-nitrobenzyl)-2'-deoxyadenosine 5'-monophosphate

NBMPR	6-[(p-nitrobenzyl)thio]-9-β-D-ribofuranosyl purine
NBMPR-P	NBMPR 5'- monophosphate
NBTGR-P	2-amino-6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine 5'-monophosphate
NEM	N-ethylmaleimide
NT	nucleoside transport
PBS	Dulbecco's phosphate-buffered saline
pCMBS	p-chloromercuribenzenesulfonate
PEI-cellulose	polyethyleneimino-cellulose
sc	subcutaneous
S.D.	standard deviation
S.E.	standard error
SITS	4-acetamido-4'-isothiocyano-2,2' disulfonic acid stibene
TCA	trichloroacetic acid
TLC	thin-layer chromatography
Urd	uridine
UV	ultraviolet

I. INTRODUCTION

A. Permeation of Small Molecules across Plasma Membranes

Animal cell membranes exhibit selective permeability to various ions and non-electrolytes of low molecular weight. Passage of nutrient and metabolite molecules across the plasma membrane of cells occur by different processes. Ellory and co-workers have provided a simple yet comprehensive classification of mechanisms involved in the transport of small solutes across plasma membranes in animal cells (Ellory *et al.*, 1988):

(a) Simple diffusion, a passive process, is driven by the concentration gradient of permeant. This process depends on permeant size, charge and lipid solubility (Hofer, 1977; Lieb and Stein, 1971; Stein, 1986). Transmembrane fluxes of small molecules such as aliphatic alcohols, urea and small sugars occur mainly by this route, especially in the non-nucleated erythrocyte (Mayrand and Levitt, 1983; Stein, 1986; Wieth, 1971).

(b) Pores and channels: There is considerable confusion in the literature as to what the terms "pores" and "channels" mean, with several workers using these terms interchangeably. Channels are generally thought to be highly selective permeation routes, mediated by membrane-spanning proteins with gating mechanisms. Examples are the sodium and calcium channels present in plasma membranes of nerve cells and cardiac myocytes (Jain, 1988). Pores may be thought of as ungated membrane discontinuities that (i) allow relatively large, usually hydrophilic molecules, to enter cells, and (ii) show limited selectivity for permeants. Some pores may have binding domains for certain molecular species that impose some permeant selectivity (Bashford *et al.*, 1988; Maier *et al.*, 1988; Nikaido and Vaara, 1985; Schafer and Barfuss, 1986). An example is the parasite-induced amino acid permeation route in erythrocytes that harbour malaria parasites (Ginsburg *et al.*, 1985; Ginsburg and Stein, 1987a,b).

(c) Facilitated diffusion processes share the equilibrative, passive properties of simple diffusion processes, but represent a higher order of complexity, being catalysed by permeant-specific, membrane carriers that mediate rapid fluxes. These processes , which can

be identified by kinetic criteria (Lieb and Stein, 1974; Stein, 1986; Widdas, 1988), are widely distributed in animal cells.

(d) Secondary active transport processes utilise energy from ionic gradients, mostly sodium and proton gradients, to accumulate unaltered substrates against their concentration gradients. Such processes are important in the influx of critical substrates such as glucose, amino acids and nucleosides into some cell types (Hofer, 1977; Paterson *et al.*, 1987; Stein, 1986).

(e) Primary active transport systems require energy input from ATP for the uphill movement of ions. Cellular sodium/potassium, calcium and proton pumps are important examples of primary active transport systems (Ellory *et al.*, 1988).

The present work investigated (i) nucleoside fluxes in neoplastic cells by the first three systems, and (ii) differences in transporter function between normal and neoplastic cells that may be exploited in chemotherapy with cytotoxic nucleosides and nucleoside transport (NT) inhibitors.

B. Nucleoside Transport in Animal Cells - An Overview

Today it is recognised that nucleoside fluxes in animal cells are mediated by nucleoside-specific membrane transport systems of which several types are known. An earlier view of nucleoside transport was that of a passive, saturable, non-concentrative process of broad substrate specificity, a classical facilitated diffusion process, for which nucleoside transport in the human erythrocyte was the model (Cabantchik and Ginsburg, 1977; Cass and Paterson, 1972; Oliver and Paterson, 1972). The sensitivity of the erythrocyte transporter to inhibition by S^6-nitrobenzyl derivatives of 6-thiopurine pentofuranosides such as NBMPR,[1] was recognised (Cass and Paterson, 1975; Paterson *et al.*, 1983b; 1985). It was shown that NBMPR was bound with high affinity to specific sites on the plasma membrane of erythrocytes and that site occupancy correlated strictly with NT inhibition (Cass *et al.*, 1974), indicating that the binding sites were part of, or associated with, transporter elements of the membrane.

[1] Abbreviations are defined on page xiv.

NBMPR binding sites have been detected on many cell types, but relationships between NBMPR binding and inhibition of NT activity in nucleated cells were found to be more complex than in erythrocytes. It has been recognised that total occupancy of the high-affinity sites by NBMPR may have no effect or may partially inhibit the influx of nucleosides in particular cell types (Belt, 1983; Dahlig-Harley *et al.*, 1981; Gati *et al.*, 1986; Paterson *et al.*, 1987). As well, some animal cell types that lack NBMPR binding sites, transport nucleosides by facilitated diffusion processes (Belt and Noel, 1985; Paterson *et al.*, 1983a).

The sodium-linked, concentrative transport of nucleosides has recently been recognised in renal and intestinal epithelial cells, in lymphoid cells and in several other cell types (Dagnino *et al.*, 1987; Jakobs and Paterson, 1986; Le Hir and Dubach, 1984; 1985; Schwenk *et al.*, 1984). Inward sodium-linked fluxes in these systems are not inhibited by NBMPR, although recent work in this laboratory (Awumey, 1989) shows that sodium-linked systems in rat hepatocytes may be NBMPR-sensitive. As well, Johnston and Geiger (1989) have described a sodium-dependent adenosine[2] transport system in a mixed population of dissociated brain cells from adult rats that appears to be sensitive to inhibition by NBMPR.

Permeation by simple diffusion, which has been seen as a minor route of nucleoside entry in some cell types (Cass *et al.*, 1981; Harley *et al.*, 1982; Jarvis *et al.*, 1982a), has been difficult to evaluate in cells with multiple NT systems. Recent findings, however, indicate that transporter-independent permeation[3] processes contribute to nucleoside fluxes to an extent that differs with cell type and permeant properties (Adjei and Paterson, 1988; Domin *et al.*, 1988; Zimmerman *et al.*, 1987).

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[2] In this report, "Ado" and "adenosine" refer to β-D-adenosine.

[3] This term refers to translocation of nucleoside molecules across the plasma membrane by mechanisms other than movement by transporter-mediated processes.

C. Carrier-Mediated Permeation of Nucleosides

Nucleoside Carriers

Carrier-mediated nucleoside fluxes in animal cells are much more rapid than diffusional fluxes. While facilitated diffusion processes are passive in the sense that they proceed without input of metabolic energy, their kinetic properties resemble those of enzyme-catalysed reactions. Such fluxes saturate, reaching a limiting value with increasing substrate concentration, and are competitively inhibited by related permeants. Other observed carrier-related characteristics of nucleoside fluxes include sensitivity to transport inhibitors, marked temperature dependence and stereoselectivity (Hofer, 1977; Paterson and Cass, 1986; Schafer and Barfuss, 1986; Wilbrandt, 1975). These transport properties may be explained by the "simple carrier model" [4] (Lieb, 1982; Stein, 1986; Widdas, 1952; Wilbrandt and Rosenberg, 1961) which assumes that biological membranes contain freely mobile[5] polypeptide "carriers" with affinity for a restricted group of substrates which they translocate across the membrane.

Facilitated Diffusion

The term "facilitated diffusion" was introduced by Danielli (1954) to refer to translocations that were not attributable to simple diffusion, but still led to equilibration, rather than accumulation of the substrate. In addition to meeting the criteria for carrier-mediated processes outlined above, facilitated diffusion processes are passive, reversible and exhibit countertransport or uphill transport (Stein, 1986; Wilbrandt, 1975).

Concentration-effect relationships for NBMPR inhibition of nucleoside transport processes in several animal cell types have provided evidence for the existence of two types of

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[4] In this model, "simple" means that only single permeant molecules are bound to the carrier (transporter), which exists in one of two conformations, each able to interact with permeant molecules at one membrane face or the other (Lieb, 1982).

[5] Carrier "mobility" refers to changes between alternative conformations of a carrier protein rather than to a physical translocation of a component of the transport system.

facilitated diffusion transport systems that may be distinguished on the basis of their sensitivity to NBMPR (Cass *et al.*, 1987; Paterson *et al.*, 1987).

NT Systems of High NBMPR Sensitivity

Cells with transporters of high NBMPR sensitivity[6] include S49 mouse lymphoma cells (Cass *et al.*, 1981), human erythrocytes, (Jarvis and Young, 1980) and guinea pig erythrocytes (Jarvis and Martin, 1986). In these cells, NBMPR binds with high affinity (K_D < 5 nM) to plasma membrane sites that are functionally associated with, or are part of the facilitated diffusion NT system. NBMPR occupancy of these binding sites correlates with NT inhibition in human erythrocytes and S49 cells (Cass *et al.*, 1974,1981). Furthermore, a single-step mutagenization of cultured S49 cells yielded the AE_1 clone, cells of which are deficient in NT activity and lack site-specific binding of NBMPR (Cass *et al.*, 1981; Cohen *et al.*, 1979). Also, "nucleoside-impermeable" sheep erythrocytes and dog erythrocytes, which lack NT activity, do not possess high-affinity NBMPR binding sites (Jarvis and Young, 1980; Jarvis *et al.*, 1982b).

The association of NBMPR binding sites with the NT system was further demonstrated by Jarvis *et al.* (1982a), who showed that the uridine (Urd) transport capacity (Vmax) of erythrocytes from several mammalian species was proportional to the abundance of NBMPR binding sites on the cells. As well, Urd and Ado, both of which are transported nucleosides, were shown to be competitive inhibitors of high affinity NBMPR binding, with apparent K_i values close to the K_m values for equilibrium exchange transport in human erythrocytes (Cass and Paterson, 1976; Jarvis *et al.*, 1982a; 1983; Koren *et al.*, 1983).

Studies that measured the temperature-dependence of changes in rates of NBMPR binding to membrane vesicles and unsealed ghosts from pig erythrocytes, demonstrated that the rate of binding to inside-out membrane vesicles was substantially reduced by a decrease in

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[6] In this study, sensitivity to NBMPR is termed high if IC_{50} (concentration of NBMPR at which nucleoside influx is reduced by 50%) values for NBMPR inhibition of cellular nucleoside transport are 5-10 nM or less, or low if the values are 1 μM or greater.

temperature from 22°C to 4°C. Similar temperature decreases, however, did not affect the rate of NBMPR binding to inside-out vesicles in the presence of detergent, or in preparations containing either right side-out vesicles or unsealed ghosts. Those results suggested that NBMPR diffusion through the erythrocyte membrane was reduced by lowering the temperature in the absence of detergents. Thus, the decrease in the rates of NBMPR binding at 4°C to inside-out vesicles, but not to right side-out vesicles, indicated that NBMPR binding sites are located on the external face of the erythrocyte plasma membrane (Agbanyo *et al.*, 1988). As well, indirect evidence has been provided in "nucleoside-permeable" sheep erythrocytes suggesting that NBMPR binding sites are located at the outer face of the plasma membrane of these cells (Jarvis *et al.*, 1982b).

The above findings are consistent with the idea that in erythrocytes and in some cultured cells, high affinity NBMPR binding activity at the plasma membrane represents interaction of NBMPR molecules with NT sites; and such binding sites appear to be located on the outer face of the plasma membrane. This interaction between NBMPR and the nucleoside permeation site has been interpreted in terms of a model in which the permeation site and the NBMPR binding site overlap (Jarvis and Young, 1982a; Jarvis, 1986; Jarvis and Young, 1987). It has been pointed out, however, that the above evidence could be accomodated in a model in which the NBMPR binding and the nucleoside permeation sites are associated, but physically distinct entities, with allosteric interactions between the permeant and inhibitor (Gati and Paterson, 1989).

Photoactivation procedures that result in covalent attachment of the ligands, ^{3}H-NBMPR or ^{3}H-azidobenzyladenosine to human erythrocyte membranes, have enabled identification of the NBMPR-binding polypeptide on electropheretograms as a band 4.5 constituent. The NBMPR binding polypeptide has an approximate Mr of 44,000-66,000 (Klip *et al.*, 1986; Young *et al.*, 1983), constitutes about 3% of the erythrocytic band 4.5 proteins[*] (Jarvis and Young, 1987), and traverses the cell membrane (Janmohammed *et al.*, 1985). Radiation-inactivation studies indicate a molecular weight of 100 - 120 kDa for the functional

[*] Nomenclature of Steck (Steck, 1974)

transporter, indicating that *in situ*, it may be a dimer (Jarvis *et al.*, 1986).

NT Systems of Low NBMPR Sensitivity

Several nucleated cells and erythrocytes of some mammalian species possess facilitated diffusion NT systems of low sensitivity to NBMPR inhibition. These cell types fall into two categories, (i) those devoid of, and (ii) those that possess high affinity NBMPR binding sites. The NT systems of both subclasses are inhibited at high concentrations ($> 1\ \mu M$) of NBMPR.

Cells without NBMPR binding sites

Some types of cultured rat neoplastic cells (such as particular clones of the Novikoff hepatoma and the Walker 256 carcinosarcoma) lack high affinity NBMPR binding sites, yet express NT systems of low NBMPR sensitivity (Paterson *et al.*, 1983a; Plagemann and Wohlhueter, 1984). Nucleoside transport processes in these cell lines seem to be similar to those in the NBMPR-sensitive systems, in being of broad substrate specificity and subject to inhibition by competing nucleoside permeants (Belt, 1983; Belt and Noel, 1985; Wohlhueter and Plagemann, 1980). Kinetic parameters for the transport of several nucleosides in these cell lines were similar to those in NBMPR-sensitive cells (Belt and Noel, 1985; Plagemann and Wohlhueter, 1984). Therefore, the absence of high affinity NBMPR binding sites in cells does not neccesarily imply the lack of a functional NT system.

Cells that possess NBMPR binding sites

Novikoff hepatoma cells of the uncloned N1S1-67 line were shown by Wohlhueter and Plagemann to be of low sensitivity to NBMPR and to lack NBMPR binding sites (Plagemann and Wohlhueter, 1984). This line, however, contained variant cells because Novikoff UA cells, a line derived from N1S1-67 stock and carried in this laboratory, expressed both NBMPR binding sites and a nucleoside transport system of low NBMPR sensitivity, as demonstrated by Gati *et al.*, (1986). Those workers demonstrated by a photoaffinity labelling

technique using ^{3}H-NBMPR that an NBMPR-binding polypeptide in a membrane-enriched preparation from Novikoff UA cells migrated on SDS gel electropherograms with an apparent Mr of 72,000-84,000. The electrophoretic mobility of the latter polypeptide is clearly different from that of the NBMPR-binding polypeptide in erythrocytes and S49 cells, which migrated with an Mr of 45,000-66,000 (Young *et al.*, 1983, 1984.). Similarly, coexpression of NBMPR-insensitive transport systems and high affinity NBMPR binding sites has been demonstrated in cells of two additional cultured rat hepatoma lines, the Morris 3924A and Reuber H-35 (Ng, 1986) lines. Photoaffinity labelling studies yielded apparent Mr values of 80,000-100,000 and 62,000-68,000, respectively, for the NBMPR binding polypeptides of the two tumours. These results suggest that NBMPR binding polypeptides from cell types in which the relation between NBMPR binding and nucleoside permeation is unclear, may be larger than polypeptides of other cell types.

NBMPR binding in Novikoff UA cells has been shown to be inhibited competitively by uridine and dipyridamole (Gati *et al.*, 1986.), suggesting that the high affinity NBMPR binding sites in these cells are in some way associated with the NT system. The other characteristics described above, however, imply that the binding sites do not couple with the permeation site as in NBMPR-sensitive NT systems, and suggest that the binding and permeation sites might be separate entities.

Joint presence of NT Systems of High and Low NBMPR Sensitivity in Cells

In a number of animal cell types that express high affinity NBMPR binding sites, total NBMPR occupancy of these sites does not result in total inhibition of NT activity. For example, Dahlig-Harley *et al.*, (1981) described biphasic relationships between NBMPR concentration and uridine transport inhibition in Hela cells. As well, Belt and Noel (1985) showed biphasic plots for the NBMPR inhibition of nucleoside transport in a cloned line of mouse leukaemia L1210 cells, and interpreted this finding to mean that these cells possess NT systems of two types, NBMPR-sensitive and NBMPR-insensitive.

Plagemann and Wohlhueter (1984) have also reported such biphasic relationships for the NBMPR inhibition of nucleoside transport in several types of cultured cells, including mouse leukaemia P388 and chinese hamster ovary (CHO) cells. More recently, Kaplinsky *et al.*, (1986) have demonstrated a similar relationship for the NBMPR inhibition of thymidine transport in two human neuroblastoma cell lines, as have Kubota *et al.*, (1988) in HL-60 cells.

In the foregoing examples, cells differed in the relative proportions of NBMPR-sensitive and insensitive nucleoside transport activities expressed. In the human lymphoblastoid cell line, RPMI 6410, the insensitive component was only about 2% of total nucleoside transport, while in HeLa cells, this component was about 40% of uridine transport (Belt, 1983; Dahlig-Harley *et al.*, 1981).

The relationship between the NBMPR-sensitive and insensitive transport systems remains unclear. The two transporters might possibly represent the products of closely related genes, or perhaps, they may be two conformations of a single protein, one which binds NBMPR with high affinity, while the other does not bind the ligand. Differences in sensitivity to the sulfhydryl reagent, p-chloromercuribenzenesulphonate (*p*-CMBS), have been observed for the two types of NT systems. The NBMPR-sensitive transporter of S49 lymphoma cells is of low sensitivity to *p*-CMBS ($IC_{50} > 400\ \mu M$), whereas the NBMPR-insensitive transporter of Walker 256 carcinosarcoma cells is of high sensitivity ($IC_{50} < 20\ \mu M$) to inhibition by *p*-CMBS, which is poorly permeable (Belt and Noel, 1985). Because cells are poorly permeable to *p*-CMBS, those results suggest that thiol groups involved in nucleoside transport in the two systems differ in their orientation in the plasma membrane, with those thiols associated with NT systems of high NBMPR sensitivity being accessible from the cytoplasmic aspect of the membrane.

Sodium-linked Nucleoside Transport

Net fluxes of nucleosides across the plasma membrane via facilitated diffusion systems are non-concentrative and occur only down permeant concentration gradients. Those systems

have been extensively studied in erythrocytes and some cultured animal cell types. In contrast, energy-dependent, concentrative NT systems have been demonstrated only recently in several cell types and exploration of these systems is at an early stage.

Ungemach and Hegner (1978) reported the uptake of thymidine in rat hepatocytes by dual systems: (i) a high affinity, active transport system with narrow substrate specificity, and (ii) a low affinity, facilitated diffusion process of broad substrate specificity. Spector and Huntoon (1982) demonstrated in rabbit choroid plexus, the operation of active transport (influx) and facilitated diffusion (efflux) systems for thymidine and deoxyuridine. The saturable efflux system was inhibitable by NBMPR without effect on the active transport system.

Le Hir and Dubach (1984,1985) described a concentrative, sodium-cotransport system for cytidine, thymidine, uridine and adenosine in rat renal brush border vesicles. This system had high affinity for the nucleosides studied (K_m values were less than 10 μM) and was of low sensitivity to NBMPR. Furthermore, Schwenk *et al.*, (1984) described an active transport system that mediated uridine entry in isolated guinea pig enterocytes, and Jakobs and Paterson (1986) reported the sodium-linked, concentrative transport of formycin B, a poorly metabolised analogue of inosine, in cultured IEC-6 rat intestinal epithelial cells. The latter system is of low sensitivity to NBMPR. Dagnino *et al.*, (1987) have described a sodium-linked transport system, in cultured mouse leukaemia L1210 cells, which in the presence of NT inhibitors, was responsible for accumulation in the leukaemia cells of free nucleosides to levels five times higher than extracellular concentrations. Such accumulation evidently occured because the inhibitors blocked the facilitated diffusion transport system that mediated nucleoside efflux across the plasma membrane, without impairing permeant influx. Vijayalakshmi and Belt (1988) have identified in mouse enterocytes two types of sodium-dependent nucleoside transporters which differ in substrate specificity. Distinguishing substrates for these transporters were formycin B and thymidine. Similar observations were made in rat intestinal epithelial cells.[1] In contrast to the NBMPR-insensitive, concentrative

[1] In cultured IEC-6 rat intestinal epithelial cells, the sodium-linked, concentrative formycin B transporter does not catalyse thymidine fluxes across the cell membrane

NT systems described above, sodium-linked, concentrative uridine and thymidine fluxes in rat hepatocytes have been found to be sensitive to NBMPR inhibition (Awumey, 1989). These findings show that sodium-linked NT systems of several types with different substrate specificities may be found in animal cells. In the latter respect, these sodium-linked transporters differ from the facilitated diffusion NT systems, which have broader substrate specificity.

It would appear from the evidence at hand that the energy-dependent NT systems may be present in transporting epithelial cells, or in cells of lymphoid origin, together with nucleoside transporter elements of the facilitated diffusion type.

D. Non-mediated Permeation of Nucleosides

Non-mediated (diffusional) fluxes of permeants across animal cell membranes are passive, non-concentrative, non-saturable fluxes that are less sensitive to temperature variations than are transporter-mediated fluxes. Diffusional fluxes are not inhibited by related permeants and are not subject to *trans* effects such as countertransport. As well, diffusional fluxes are dependent on the lipophilicity and molecular size of permeant (Nikaido and Rosenberg, 1981; Stein, 1986; Hofer, 1977; Goldenberg and Begleiter, 1984). The hydrophilic nature of the physiological nucleosides led to the conclusion that diffusional processes did not contribute significantly to their fluxes across animal cell membranes (Berlin and Oliver, 1975). Supporting evidence has included demonstrations that in several tumour cell clones selected for resistance to cytotoxic nucleosides, defective facilitated diffusion NT systems appear to impart resistance (Cass *et al.*, 1981; Cohen *et al.*, 1985; Sobrero *et al.*, 1985). Also, cultured cells have been protected from otherwise cytotoxic concentrations of nucleoside analogues by the presence of NT inhibitors in culture media (Warnick *et al.*, 1972; Paterson *et al.*, 1979a). Wiley *et al.* (1983), showed that at therapeutic concentrations of araC (1μM), about 90% of the influx into human leukaemic lymphoblasts was carrier-mediated.

[1](cont'd) (Jakobs, E. S., and Paterson, A. R. P., unpublished results).

Diffusional fluxes of nucleosides, however, have been observed in some animal cell types. Roos and Pfleger (1972) showed that adenosine permeation in guinea pig erythrocytes included, (i) a facilitated diffusion, dipyridamole-sensitive component, and (ii) a non-saturable, dipyridamole-resistant process. The latter process also contributed to the cellular uptake of inosine, N^6-methyl and dimethyl derivatives of adenosine, and fluxes of these agents correlated with their octanol-water partition coefficients. Jarvis and Martin (1986) have confirmed this earlier study by describing a non-mediated component of uridine influx in guinea pig erythrocytes. In his studies of nucleoside transport in sheep erythrocytes, Young (1978) showed that erythrocytes from most sheep were virtually impermeable to 5 mM inosine (nucleoside-impermeable phenotype), while cells from approximately 5% of the animals exhibited rapid inosine influx (nucleoside-permeable phenotype). Cells from both types of animal were permeable to 5 mM adenosine, although fluxes were slower in nucleoside-impermeable erythrocytes. Two nucleoside permeation pathways were apparent in nucleoside-permeable erythrocytes, a facilitated diffusion system transporting purine and pyrimidine nucleosides, and a non-saturable influx route. Dipyridamole and a large molar excess of inosine had no effect on the non-saturable component of adenosine influx into sheep erythrocytes. More recently, Zimmerman *et al.*, (1987) and Domin *et al.*, (1988) have reported the non-mediated influx of 3'-azido-3'deoxythymidine (AZT) and 2',3'-dideoxythymidine in human erythrocytes and lymphocytes. This mode of influx was attributed to the greater lipid solubility of AZT and 2'3',-dideoxythymidine relative to that of thymidine.

In human erythrocytes, the intracellular presence of *Plasmodium falciparum* has been shown to lead to non-saturable fluxes of several solutes, including amino acids, hexoses and polyols (Ginsburg *et al.*, 1987a,b; Kutner et al., 1983, 1985; Sherman, 1988). Increased influx of nucleosides has been observed in *P. falciparum*-infected erythrocytes. In contrast to the native nucleoside transporter of the erythrocyte, the parasite-induced permeability pathways are of low sensitivity to NT inhibitors, but it is not yet known if the induced fluxes are transporter-mediated (Gero *et al.*, 1988). As well, two new, parasite-induced pathways of

adenosine influx have been described in *Plasmodium yoelii*-infected mouse erythrocytes. One component of Ado influx is saturable, whereas the other component appears to be non-saturable, and is not enantioselective. Both permeation processes are insensitive to NBMPR inhibition, but the latter system is inhibited by furosemide with an IC_{50} of approximately 15 μM (W. P. Gati *et al.*, manuscript in preparation).

E. Transport and Metabolism of Nucleosides

Transport is the initiating step in the multi-step process of nucleoside accumulation in animal cells. "Transport" and "permeation" in this work will refer to the passage of permeant molecules across the plasma membrane, and "uptake" will refer to the cellular accumulation of a nucleoside permeant and its metabolic products, which may include the mono-, di- and triphosphoesters, and, in some instances, incorporation into polynucleotides (Paterson and Cass, 1986).

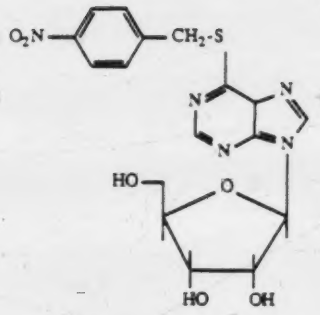
Nucleoside uptake in many permeant-metabolising cells proceeds by two tandem, independent processes, (i) transport or permeation which may take place by several mechanisms some of which are reversible, and (ii) kinase-mediated phosphorylation steps. In studying uptake in a number of cultured cell systems, several workers have concluded that the transport carrier-substrate affinity constants and the maximum velocities of the transport systems of virtually all physiological nucleosides considerably exceed the corresponding values for the phosphorylation reactions (Heichal *et al.*, 1979; Koren *et al.*, 1979; Lum *et al.*, 1979; Plagemann *et al.*, 1978; Wohlhueter *et al.*, 1976). Thus, transport and phosphorylation capacities determine the overall nucleoside uptake rate. Transport rate is the major determinant of uptake at low substrate concentrations, while phosphorylation rate is the major determinant at high substrate concentrations (Plagemann and Wohlhueter, 1980).

Nucleoside transport is rapid in many animal cell types, and rapid sampling methods are needed to obtain time courses of nucleoside influx that accurately define initial rates. Regardless of the complexity of the time course of permeant uptake by cells, the initial rate of the uptake process is intrinsically the rate of influx of the permeant (Paterson and Cass,

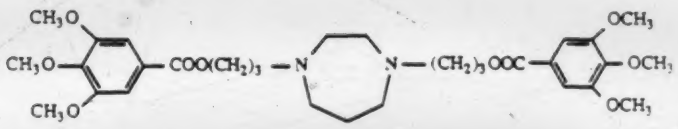
1986).

F. Inhibitors of Nucleoside Transport

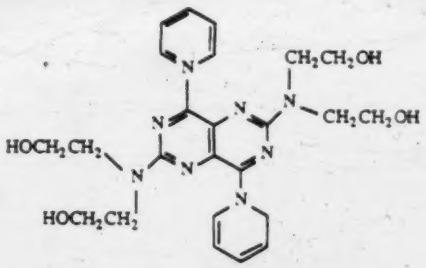
Several pentofuranosides of S^6-substituted 6-thiopurines and N^6-substituted adenine derivatives are potent NT inhibitors in a variety of normal and transformed animal cells (Cass *et al.*, 1981; Paterson *et al.*, 1983a,b). Nitrobenzylthioinosine (NBMPR, Fig. 1), is one of the most potent and the best studied of the NT-inhibitory 6-thiopurine derivatives. NBMPR binds tightly (K_D, 0.1-5 nM) to sites that are associated with, or are part of the facilitated diffusion NT elements of the plasma membrane of various cell types (Cass *et al.*, 1974). The relationship between the NBMPR binding site and the nucleoside permeation site has been a matter of discussion in the recent literature (Gati and Paterson, 1989; Jarvis, 1986; Jarvis and Young, 1987; Paterson *et al.*, 1987). In recent years, two types of facilitated diffusion NT mechanisms have been identified that are distinguishable on the basis of NBMPR sensitivity. Concentrative, sodium-dependent NT systems have also been demonstrated recently in some cells. The present study describes a transporter-independent nucleoside permeation system. The latter system and some of the sodium-dependent NT systems are also not inhibited by NBMPR (Adjei and Paterson, 1988; Paterson *et al.*, 1986). NBMPR has been a very useful probe for the characterisation of the function, biology and structure of NT systems in animal cells, and as a modulator of the cytotoxicity of nucleosides in experimental cancer chemotherapy studies. The hydrophobic nature of the S^6 -substituent in these compounds is thought to contribute to the interaction between the inhibitor and the transport -inhibitory site (Paul *et al.*, 1975). In S49 mouse lymphoma cells, NBTGR inhibits



NITROBENZYLTHIOINOSINE



DILAZEP



DIPYRIDAMOLE

Fig. 1. Structural formulae of three potent NT inhibitors.

adenosine transport with an IC_{50} of 0.1 nM, compared to the NBMPR value of 1.7 nM (Paterson *et al.*, 1983b). N^6-substituted adenine nucleosides, notably N^6-nitrobenzyldeoxyadenosine (NBdAdo), also interacts strongly with the NBMPR binding site in animal cell membranes. The IC_{50} for NBdAdo inhibition of adenosine transport in S49 cells is 4 nM (Paterson *et al.*, 1983b).

NBMPR in Studies of the Cell Biology of NT Processes

NBMPR binding polypeptides comprise less than 0.5% of the membrane proteins in human erythrocytes (Young, *et al.*, 1983). Radiolabelled ligands with high affinity for the binding site have been important in attempts to isolate and characterise the NBMPR-binding polypeptides. Exposure of cell or membrane preparations with site-bound NBMPR or congeners to ultraviolet (uv) light results in covalent attachment of the site-bound ligand to the binding polypeptides. ^{3}H-NBMPR has been a useful tool in such photoaffinity labeling studies (Janmohammed *et al.*, 1985; Wu *et al.*, 1983; Young *et al.*, 1984). Photolabeling with ^{3}H-NBMPR has allowed comparison of the relative molecular weights of NBMPR binding polypeptides in different cell types. Binding polypeptides from rabbit and mouse erythrocytes, rat lung, guinea pig heart, lung and liver are similar in size (Mr, 55,000-60,000) to the binding polypeptide from human erythrocytes (Gati *et al.*, 1987; Jarvis *et al* ., 1986; Kwan and Jarvis, 1984; Shi *et al.*, 1984; Young and Jarvis, 1985), suggesting similar nucleoside transporters in these cells. Similar results have been obtained with membrane preparations from mouse lymphoma S49 cells (Almeida *et al.*, 1984; Young *et al.*, 1984).

The NBMPR binding polypeptides from some lines of cultured rat hepatoma cells (Novikoff UA, Reuber H35, Morris 3924A; Mr, 72,000-80,000) are larger than those from the cell types described above. The hepatoma cells possess NT systems of low sensitivity to NBMPR, despite the presence of high-affinity binding sites.

G. Non-nucleoside Inhibitors of Nucleoside Transport

In addition to NBMPR and its congeners, a number of vasodilators with no obvious structural resemblance to NBMPR have been recognised as potent inhibitors of nucleoside permeation and NBMPR binding in animal cells. This group includes dipyridamole (Lum *et al.*, 1979), dilazep (Pohl and Brock, 1974), hexobendine (Kolassa *et al.*, 1978), lidoflazine (Van Belle, 1970), and mioflazine, together with related compounds (Van Belle, 1988).

Dipyridamole, the best studied among these drugs, has been shown to be a competitive inhibitor of inward transport of uridine in erythrocytes from guinea pigs and sheep, (K_i, 1 nM), but a non-competitive inhibitor of uridine efflux in these cells. (Jarvis *et al.*, 1982b; Jarvis, 1986). As well, Jarvis (1986) demonstrated in human erythrocyte membranes that ^{3}H-dipyridamole was bound by a single class of high-affinity sites (K_D, 0.65 nM), present in these cells in numbers similar to those of NBMPR binding sites. Such binding was inhibited by adenosine, uridine, NBMPR, NBTGR and dilazep. In other studies, dipyridamole inhibited the site-specific, high-affinity binding of NBMPR to human and rat erythrocytes (Hammond *et al.*, 1981; Jarvis, 1986). These observations have been interpreted to mean that in erythrocytes with NBMPR-sensitive systems, dipyridamole and NBMPR bind at overlapping sites on the external face of the plasma membrane, and that this site, wholly or partially, includes the permeation site (Jarvis, 1986). It has been suggested that this interpretation overlooks the structural dissimilarities between uridine, NBMPR, dipyridamole and dilazep (Gati and Paterson, 1989).

Exploration of concentration-effect relationships for NBMPR inhibition of NT in several cultured cell lines, including HL-60, HeLa, L1210 and CHO, has revealed biphasic relationships that indicate the joint presence in cells of NT systems of both high and low sensitivity to NBMPR. However, similar studies in these cells with dipyridamole have yielded monophasic curves (Belt, 1983; Kubota *et al.*, 1988; Paterson *et al.*, 1980; Woffendin and Plagemann, 1987). Furthermore, dipyridamole inhibited NT in Walker 256 cells, which are devoid of NBMPR binding sites, indicating that dipyridamole does not distinguish between transporters of high and low sensitivity to NBMPR. The foregoing results suggest that the

determinants of NBMPR and dipyridamole interaction with NT systems are different. In addition to inhibition of nucleoside transport, dipyridamole also inhibits transport of nucleobases, hexoses, choline and phosphate. (Plagemann and Richey, 1974; Van Belle, 1988).

Dilazep hydrochloride has high aqueous solubility and has been employed as a transport-stopping agent in the measurement of nucleoside fluxes in cells with NBMPR-sensitive transporters (Paterson *et al.*, 1984). In such cells, dilazep has been shown to inhibit NT activity with IC_{50} values of 5-100 nM (Mahony and Zimmerman, 1986; Paterson *et al.*, 1983; Plagemann and Kraupp, 1986).

Other inhibitors of nucleoside transport include cytochalasin B, the epipodophyllotoxins (e.g., teniposide (VM-26) (White *et al.*, 1985), and papaverine (Plagemann and Wohlhueter 1980). Some calcium channel antagonists, such as verapamil and nifedipine, are also weak NT inhibitors (Plagemann and Woffendin 1986). These compounds probably inhibit nucleoside transport by interacting with cellular components different from those with which NBMPR and its congeners interact.

NT inhibition by thiol reagents implicates thiol groups in the transport process (Eilam and Cabantchik 1977). Belt and co-workers found that *p*-CMBS, a thiol reagent that penetrates membranes poorly, inhibited NT systems of low NBMPR sensitivity in L1210 leukaemia and Walker 256 cells ($IC_{50} < 25\ \mu M$), but did not inhibit the NT system of high NBMPR sensitivity in S49 cells ($IC_{50} > 400\ \mu M$) (Belt, 1983; Belt and Noel, 1985). In later studies, Jarvis and Young (1986) demonstrated that in rat erythrocytes, *p*-CMBS inhibited a uridine influx process of low NBMPR sensitivity, but had little effect on the NBMPR-sensitive component of uridine transport. Furthermore, *p*-CMBS had no effect on NBMPR binding to intact rat erythrocytes, but inhibited NBMPR binding to disrupted membranes. Together, these results indicate that essential thiol groups in nucleoside transporter polypeptides are in some way involved in transportef function and that, in cells with NT systems of low NBMPR sensitivity, such thiol groups are accessible from the outer face of the membrane, while in cells with NT systems of high NBMPR sensitivity, those groups are accessible from the cytoplasmic face of the membrane.

In studies at 37°C with L1210 and Walker 256 cells, Plagemann and Wohlhueter (1984) did not detect differences in *p*-CMBS inhibition of the NBMPR-sensitive and insensitive NT systems, indicating that the differential effects of *p*-CMBS may be evident only at temperatures that do not allow significant permeation of the thiol reagent across the plasma membrane.

H. Nucleoside Analogues in Cancer Chemotherapy

Nucleoside analogues comprise a group of compounds that differ from the physiological purine and pyrimidine nucleosides in structural features in the base and/or sugar portions of the molecule. Some analogues are natural compounds obtained from bacteria and fungi, and a great number have been synthesised in attempts to develop new chemotherapeutic agents or to improve upon existing ones. Many of these agents possess potent biological activities including cytotoxicity and have been used as antibacterial, antiviral and antineoplastic agents. (for reviews, see Suhaldonik 1979, and Robins, 1985). A number of such analogues have established roles in anticancer or antiviral chemotherapy and others show considerable promise and are under active investigation.

Cytosine arabinoside (araC), the most widely used anticancer nucleoside analogue, is the cornerstone of therapy for adult non-lymphocytic leukaemia (Muggia and Carter, 1987). 5-Azacytidine and 5-fluorodeoxyuridine are used in treatment of refractory acute myeloid leukaemias and gastrointestinal neoplasms, respectively (Krakoff, 1987; Muggia and Carter, 1987). Various nucleoside analogues, including triciribine and its 5'-monophosphate, dideoxycytidine, 2'-deoxycoformycin, fludarabine phosphate and 3-deazauridine, are under active investigation as anticancer agents either as single agents, or in combination with other agents (NCI, 1988; O'Dwyer *et al.*, 1987; Schilcher *et al.*, 1986). In addition, interest in some "old" nucleoside antibiotics has been rekindled (Ritch and Glazer, 1984). New approaches to the use of these toxicants, such as the use of tubercidin in marrow-purging procedures prior to autologous bone-marrow transplantation, are under study (Cass and Janowska-Wieczorek, 1988).

I. The Host-protection Tactic: Combination of NT Inhibitors with Cytotoxic Nucleosides

Clinically useful anticancer drugs are commonly limited by low therapeutic indices and poor selectivity for tumour cells relative to normal tissue. A substantial research effort has been aimed toward increasing the therapeutic indices of cytotoxic agents through biochemical modulation. One of the approaches employed is the protection of normal host tissues from drug toxicity by other agents, which could be neutralising agents, enzyme inhibitors or possibly transport inhibitors (Leyland-Jones *et al.*, 1986).

NT inhibitors have been shown to reduce the antiproliferative activity of cytotoxic nucleoside analogues towards various types of neoplastic cells in culture. For example, inclusion of 5 μM NBMPR in growth media protected RPMI 6410 cells from the antiproliferative effects of 5-azacytidine, araC, 2-F-Ado and showdomycin (Paterson *et al.*, 1979b). The growth-inhibitory effects of araC on HL-60 cells in culture was decreased by the presence of 1 μM dipyridamole in culture media (King *et al.*, 1984). Following such *in vitro* observations, Paterson *et al.*, (1979a) and Lynch *et al.*, (1981a) demonstrated the protection of mice by NBMPR and by NBMPR 5'-monophosphate (NBMPR-P)[9] against potentially lethal doses of nebularine and tubercidin. When mice implanted with leukaemia L1210/TG8 were treated with lethal doses of nebularine plus optimised, host-protecting doses of NBMPR-P, the combination achieved a substantial leukaemic cell kill, demonstrating that dose-limiting tissues were better protected than the leukaemic cells (Lynch *et al.*, 1981a). Treatment with tubercidin-NBMPR-P combinations similarly achieved selective toxicity against three other transplantable murine tumours (colon 26 carcinoma, the Ehrlich ascites carcinoma, and leukaemia L1210/TG8) (Lynch *et al.*, 1981b). These findings suggested that neoplastic cells with NBMPR-insensitive nucleoside transporters might be responsive to chemotherapy with potentially lethal doses of nucleoside analogues, administered together with host-protective doses of NBMPR-P. In testing this strategy in chemotherapy experiments with the Walker 256 rat carcinosarcoma, (i) male Sprague-Dawley rats were protected from

[9] NBMPR-P, a prodrug form of NBMPR, has greater aqueous solubility than the parent compound. While NBMPR-P *per se* has no NT inhibitory activity, the pro-drug is rapidly dephosphorylated in the circulation to NBMPR (Ogbunude *et al.*, 1984)

otherwise lethal doses of tubercidin by coadministration of NBMPR-P, and (ii) treatment of tumour-bearing rats with the tubercidin-NBMPR-P combination cured a high proportion of the treated animals. This tactic has also been employed with some success in the experimental therapy of several parasitic diseases. Combinations of tubercidin or nebularine with NBMPR-P have been used in treating experimental schistosomiasis (El Kouni *et al.*, 1983; 1987), trypanosomiasis (Ogbunude and Ikediobi, 1982), and malaria (Gati *et al.*, 1987). In those experiments, both toxicant and protectant were administered intraperitoneally to the rodents.

"Two-route" Chemotherapy

A theoretical advantage of intraperitoneal (ip) administration of antineoplastic drugs is the possibility of concurrently using systemically delivered antidote, inhibitor or neutralising agent. The ideal modulating agent would protect normal host tissues from toxicity of the anticancer drug, and yet allow the local instillation of high dosages of the latter. This approach has been called "two-route" chemotherapy (Baba *et al* ., 1980).

Peritoneally-disseminated neoplastic cells in mice and rats have been successfully treated with ip cisplatin and subcutaneous (sc) sodium thiosulphate (Taniguchi and Baba, 1982; Iwamoto *et al.*, 1984). As well, the safety and efficacy of this tactic has been demonstrated in clinical trials with two toxicant-protectant pairs, methotrexate-folinic acid (Howell *et al.*, 1981; Jones *et al.*, 1981) and cisplatin-thiosulphate (Howell *et al* ., 1982; 1983). Toxicants were administered intraperitoneally with concurrent intravenous administration of protectants. This approach was investigated in the present study, using ip cytotoxic nucleosides and sc NT inhibitors.

J. Ovarian Carcinoma

Carcinoma of the ovary is the seventh leading cancer in women, and accounts for four percent of all cancers prevalent in females in North America. It is the leading cause of gynaecologic cancer deaths, accounting for forty-nine per cent of such deaths (Silverberg and

Lubera, 1989). These neoplasms are difficult to diagnose, and patients typically remain asymptomatic until the disease is far advanced. Advanced tumours comprise 60 to 70 per cent of cases at initial diagnosis. Survival rates for patients with ovarian cancer are still poor; only 5-25% of patients with invasive cancer survive five years or longer (Ozols and Young, 1987; Slotman and Rao, 1988). The current best treatment for this disease comprises a combined approach of surgical debulking followed by cytotoxic combination chemotherapy with cisplatinum-based regimens. Clinical response rates of 80-90%, including 40% complete responses have been obtained (Thigpen *et al.*, 1984; Barber, 1986, Slotman and Rao, 1988). In spite of these high response rates, recurrence among disease-free individuals remains high (20-40%) (Cain *et al.*, 1986; Thigpen *et al.*, 1988).

Approximately 80 to 90 percent of primary ovarian carcinomas arise from the coelomic (surface) epithelium, and include serous, mucinous, endometroid, clear-cell and undifferentiated adenocarcinomas (Robbins *et al.*, 1984). These neoplasms tend to spread by surface implantation and remain in the peritoneal cavity for most of their natural history, and thus lend themselves to intraperitoneal chemotherapy (D'Acquisto *et al.*, 1988). For that reason, ovarian carcinoma was chosen as the human model for investigating intra-cavitary chemotherapy.

Several workers have attempted to purify human carcinoma cells from ascitic fluids of patients for *in vitro* studies (Hamburger *et al.*, 1984; Minami *et al.*, 1978; Mantovani *et al.*, 1980). Such attempts have been only partially successful. In our experience, tumour cell yields have been variable, and contamination with mesothelial cells was common. The problem of obtaining "pure" carcinoma cells in large quatities for biochemical assays was overcome by the use of cultured ovarian carcinoma cells.

K. Nucleoside Analogues of Importance in this Study

Nebularine

Nebularine (9-β-D-ribofuranosylpurine), was first isolated by Ehrenberg and co-workers in 1946 from the mushroom, *Agaricus nebularis*. Nakamura (1961) later reported the isolation of this agent from a streptomyces species. This nucleoside is toxic to human bovine and avian strains of *Mycobacterium tuberculosis*, sarcoma 180 ascites tumour cells both in culture and in vivo, embryonic fibroblasts and epithelial cells of embryonic skin (Biesele *et al.*, 1955). Nebularine is very toxic to mice and rats and this property discouraged early study of the antineoplastic properties of this agent. The aglycone (purine) is relatively non-toxic (Brown and Weliky 1953).

Nebularine is transported into cultured L1210 cells where it is converted into the mono-, di-, and triphosphate esters and thus trapped internally (Lynch *et al.*, 1981a). The initial phosphorylation appears to be accomplished by adenosine kinase (Lindberg *et al.*, 1967). The cytotoxic effects of nebularine appear to be mediated through pertubations in intermediary metabolism by intracellular mono-, di-, and triphosphate derivatives of the agent, but the exact biochemical events are unknown (Lynch *et al.*, 1981a).

Tubercidin

Tubercidin, 6-amino-9-(β-D-ribofuranosyl)-7-deazapurine, was isolated from culture filtrates of *Streptomyces tubercidus* in 1957. This adenosine analogue is toxic toward many types of cultured mammalian cells, including NF mouse sarcoma cells, mouse fibroblasts, DON cells, chinese hamster ovary and HeLa cells, among others (Ritch and Glazer, 1984). Owen and Smith (1964) demonstrated that tubercidin had activity against sarcoma 180 ascites tumour cells, Ehrlich ascites tumour cells, Jensen sarcoma cells and Dunning ascites leukaemia cells growing in rodents.

Bisel *et al.*, (1970) showed in a phase I clinical trial of tubercidin administered by intravenous injection that significant toxicity was limited to the kidneys, and that severe tissue

necrosis resulted from extravasation of the agent. Three responses in patients with islet cell carcinoma of the pancreas were reported. Grage *et al.*, (1970) continued the phase I studies with tubercidin administered after uptake by erythrocytes, to eliminate the severe tissue irritation. A significant antitumour effect was observed in four patients, three with islet cell carcinoma of the pancreas and the other with a carcinoid tumour of the stomach. While further clinical studies with tubercidin have not been reported, this drug might have a place in the treatment of gastrointestinal tumours, if toxicity was manageable.

The bases of tubercidin cytotoxicity have not been defined, but it is known that tubercidin is (i) a substrate for the facilitated diffusion NT mechanism in animal cells (Irish *et al.*, 1979), (ii) a substrate for adenosine kinase (Lindberg *et al.*, 1967), (iii) anabolised intracellularly to mono-, di- and triphosphate esters (Parks and Brown, 1973), and (iv) incorporated into RNA and DNA (Suhaldonik, 1979). Tubercidin is not a substrate for adenosine deaminase nor for purine nucleoside phosphorylase. The biochemical sites at which tubercidin metabolites interfere with physiological processes in the expression of cytotoxicity remain unidentified.

Cytosine Arabinoside

β-D-arabinofuranosylcytosine (araC) is one of the most effective drugs for the treatment of adult acute myelogenous leukaemia (Frei *et al.*, 1969), and is probably the most widely studied and clinically utilised of the cytotoxic nucleosides in cancer chemotherapy.

AraC is an analogue of deoxycytidine, first synthesised in 1950, and introduced into clinical medicine in 1963 (Fonken, 1970). AraC is S-phase specific (Bhuyan *et al.*, 1972) and the triphosphate, araCTP, is an inhibitor of DNA synthesis in a variety of viral, bacterial and mammalian cell systems (Cohen, 1966; Woodcock, 1987). Because of rapid deamination in the liver, plasma, kidney and gastro-intestinal tract to the virtually non-toxic catabolite, uracil arabinoside, S-phase specificity and rapid excretion, araC is administered clinically by continous infusion for 5-10 day intervals.

The biochemical mechanisms responsible for the drug's cytotoxic effects have been unclear. Furth and Cohen (1968) and Graham and Whitmore (1970) had indicated that araCTP inhibited DNA polymerase by competing with the binding of dCTP to this enzyme, while more recent findings by Major *et al.*, (1981), Kufe and Major (1982), and Kufe and Spriggs (1985) have shown that araC is incorporated into DNA. The incorporated araC residues are thought to serve as poor termini for chain elongation, thereby resulting in inhibition of DNA synthesis. However, using time-lapse photography to study cells exposed to araC, Crowther *et al.*, (1985) have shown that the lethally injured cells progress through multiple cell divisions before death. These findings imply that araC incorporation into DNA does not abruptly terminate cell division. Other mechanisms of cell injury evidently contribute to produce lethality (Woodcock, 1987).

II. INTRODUCTION TO PRESENT STUDY

This laboratory has long-standing interests in nucleoside transport in animal cells. Recognition that NT systems expressed in mammalian cells may differ in NBMPR sensitivity from one cell type to another led to the evolution of the host-protection tactic in cancer chemotherapy. In this tactic, drug treatments employ combinations of cytotoxic nucleosides and NT inhibitors. The combination of NBMPR with otherwise lethal dosages of tubercidin or nebularine led to substantial rates of neoplastic cell kill and to cures in tumour-bearing mice, evidently because NBMPR blocked the influx of the toxicants into dose-limiting host tissues without doing so in target cells.

In the present study, the use of several potent NT inhibitors in the host-protection tactic in cancer chemotherapy was explored. The NT inhibitors investigated were dilazep and dipyridamole, which are non-nucleoside drugs, and NBMPR-P, NBdAdo-P and NBTGR-P, which are pro-drug forms of NBMPR and congeners. Routes of inhibitor administration other than the ip route previously utilised were also investigated.

Understanding the transport mechanism of physiological nucleosides into animal cells is fundamental to understanding the transmembrane permeation of cytotoxic nucleoside analogues, since cellular NT systems are major routes of entry of such analogues into cells (Paterson and Cass, 1986; Paterson *et al.*, 1987). In recent years, the diversity of NT mechanisms in animal cells has been recognised (Paterson *et al.*, 1987). This has necessitated the characterisation of the NT system(s) in human neoplastic cell types, as a first step in applying the above chemotherapeutic strategy to the treatment of human neoplastic disease. Human ovarian carcinoma was chosen for investigation because (i) it is an important cause of neoplastic morbidity and mortality, (ii) growth is typically confined to the peritoneal cavity (which is ideal for ip chemotherapy), and (iii) treatment results at present, especially in advanced cases, are not satisfactory (Slotman and Rao, 1988)

III. MATERIALS AND METHODS

A. Materials

NBMPR was prepared in this laboratory by an established method (Noel and Robins, 1962), using as starting material, 6-thioinosine generously provided by the Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. NBMPR-P, NBTGR-P and NBdAdo-P were prepared as the disodium salts by the research laboratory of Yamasa Shoyu Ltd. Chosi, Japan. Dipyridamole and dilazep were gifts from Boehringer-Ingelheim (Canada) Ltd. (Dorval, Quebec) and F. Hoffmann-La Roche and Co. (Basel, Switzerland). AraC, tubercidin, 3-deazauridine, 5-azacytidine and β-L-adenosine were provided by the Drug Synthesis and Chemistry Branch, DCT, NCI, Bethesda, MD. All other unlabelled nucleosides were from Sigma Chemical Co. (St. Louis, Mo). Tricaprylylamine (alamine 336) and 1,1,2-trichlorotrifluoroethane (Freon TF) were obtained, respectively, from the Henkel Co. Kankakee, IL., and from Dupont, Maitland, Ont. PEI cellulose TLC sheets were from Brinkman Instruments, Westbury, New York.

Radiochemicals

[G-^{3}H]NBMPR (35 Ci/mmol), [2,8-^{3}H]adenosine (23 Ci/mmol), [G-^{3}H]formycin B (3 Ci/mmol), [5-^{3}H]uridine (20 Ci/mmol), [methyl-^{3}H]thymidine (60 Ci/mmol), [8-^{3}H]guanosine (11 Ci/mmol), [2-^{3}H]inosine (16 Ci/mmol) and [8-^{3}H]-L-adenosine (33 Ci/mmol) were obtained from Moravek Biochemicals, Brea, CA. ^3H_2O (100 mCi/ml) was purchased from ICN , Irvine CA, [1,2-^{3}H]polyethylene glycol (2mCi/g) was from New England Nuclear, Boston, MA, and [U-^{14}C]sucrose (671 mCi/mmol) was from Amersham, Arlington Heights, IL. Radiochemicals were purified by high pressure liquid chromatography to greater than 98% radiochemical purity, using a C_{18} μ Magnum 9 column (Whatman, Clifton, NJ), eluted with methanol/water solutions.

Cell Culture Materials

Stocks of SK-OV-3 cells, a human ovarian adenocarcinoma cell line, were obtained from the American Type Cell Culture Collection (Rockville, MD). Frozen stocks of HOC-7, HOC-1 and HEY cells, other human ovarian adenocarcinoma cell lines, were generously provided by Dr. R. N. Buick (Ontario Cancer Institute). All cell culture materials were supplied by Gibco Laboratories (Burlington, Ontario).

Animals

Male and female C57BL/10J x DBA/2J F_1 hybrid mice, hereafter termed $B6D2F_1$ mice, were purchased from the Health Sciences Animal Service, University of Alberta. Mouse leukaemias L1210, P388 and Erhlich ascites carcinoma cells for chemotherapy experiments were obtained from frozen stocks in this laboratory.

B. Cell Culture

SK-OV-3 cells were grown as monolayers in McCoy's 5A medium supplemented with 15% foetal bovine serum in a 5% CO_2-air atmosphere at 37° in a humidified incubator. The cell population doubling time was about 48 h. These cells were later adapted to growth in α-MEM medium supplemented with 10% foetal bovine serum. Under these culture conditions, the cell population doubling time was about 28 h.

HOC-7, HEY and HOC-1 cells were grown as monolayers in α-MEM medium supplemented with 10% foetal bovine serum in a 5% CO_2-air atmosphere as above. Cell population doubling times were 20 h, 17 h and 40 h respectively. All monolayers were grown in 80 cm^2 flasks (Nunc, Denmark). To maintain cells in exponential growth, SK-OV-3 and HOC-1 stock cultures were subcultured weekly, and HOC-7 and HEY cells were subcultured twice weekly, using inocula of 2-5 x 10^3 cells/ml for all lines. When cells were propagated for experimental work, culture media contained penicillin G, 100 units/ml and streptomycin 100 μg/ml. Frozen stocks were mycoplasma-free (Dr. J. A. Robertson, Department of Medical Microbiology, University of Alberta), and stock cultures were re-started from frozen stock at

8-10 week intervals. Confluent monolayers were used for experiments.

C. Binding Studies

Assays for equilibrium binding of ^{3}H-NBMPR were used to determine the numbers of high affinity NBMPR binding sites on SK-OV-3 and HOC-7 cells. Total binding was determined from the cellular ^{3}H-content after incubating the monolayers for 20 minutes at room temperature (22°) with graded concentrations of ^{3}H-NBMPR. Non-specific binding was determined from the cellular ^{3}H-content in similar experiments with assay mixtures containing 10 μM non-isotopic NBMPR. Specific binding was defined as the difference between total and non-specific binding (Cass *et al.*, 1981). These experiments employed replicate monolayer cultures with 1-2 x 10^{6} cells per plastic culture dish (35mm for SK-OV-3 cells, 60mm for HOC-7 cells), and assays were conducted in triplicate.

Intervals of cell-ligand equilibration were terminated by removing the permeant-containing medium by suction from the culture dishes, after first removing 100 μl samples for assay of free ^{3}H-NBMPR concentrations in the incubation mixture, and monolayers were immediately and rapidly rinsed five times with 2-ml portions of ice-cold phosphate buffered saline (PBS) (Dulbecco and Voght, 1974). Dishes were individually processed through the above procedures that terminated intervals of equilibration. Rinsing was carried out with the culture dishes placed on an angled, ice-cold metal tray and the PBS was removed by continous aspiration as it flooded over the monolayer (Heichal *et al.*, 1978). The monolayers were drained by inversion on absorbent paper and 0.75 ml of 5% Triton X-100 (v/v) was added to each SK-OV-3 culture dish; HOC-7 monolayers received 1.25 ml of 5% Triton x-100. Dishes were kept at 37° in a humidified incubator for 45 minutes to promote solubilization. To determine ^{3}H-content, portions of the solubilized samples (0.5ml, SK-OV-3; 1.0 ml, HOC-7) were mixed with 8 ml of a Triton-X-100-xylene scintillation fluid (Pande, 1976) and assayed for ^{3}H-activity by liquid scintillation counting. Mass law analysis of the binding data was conducted with the LIGAND computer programme (Munson and Rodbard, 1980).

D. Adenosine Influx Measurements

Kinetics of Adenosine Transport

Ado fluxes in animal cells are rapid, and time courses of cellular uptake of adenosine over brief intervals (0-15 s) must be employed in order to define initial rates of adenosine uptake; initial rates of permeant uptake measure unidirectional inward fluxes. In this study, a minor modification of the rapid sampling technique developed by Hawkins and Berlin (1969) was employed to measure rates of nucleoside uptake in monolayer cultures of ovarian carcinoma cells.

Replicate monolayer cultures (1-2 x 10^6 cells/dish), were exposed at 22° for graded intervals, to particular concentrations of ^{3}H-Ado in "transport medium", which for SK-OV-3 cells, was McCoy's medium without serum and bicarbonate, but supplemented with 20 mM HEPES (pH 7.4 at 22°). For other cell lines, transport medium consisted of α-MEM medium modified and supplemented in the same way. Intervals of uptake were started by flooding SK-OV-3 monolayers with 0.75 ml of adenosine-containing medium (1.5 ml for other cells), and were terminated by (i) rapidly aspirating most of the medium about 1 s before the end of a designated time interval, and (ii) immersing the dish in a two-litre volume of ice-cold PBS in response to a metronome signal ending that interval. Culture dishes further were rinsed, rapidly and consecutively, in two beakers each containing one-litre volumes of ice-cold PBS each. The dishes were then tapped lightly to dislodge PBS drops, inverted over absorbent paper and allowed to dry.[10] SK-OV-3 monolayers were then dissolved in 0.75 ml (1.25 ml for other cells) of 5% Triton X-100. The procedure was hastened by placing dishes for 30-45 min at 37° in a humidified incubator, and 0.5 ml samples of the SK-OV-3 lysates (1.0 ml lysates for other cells) were then assayed for radioactivity.

The loss of labelled Ado during the cold rinses was determined to be negligible by washing replicate monolayer cultures for graded intervals and comparing cellular content of labelled permeant. Furthermore, untreated monolayers passed through the rinse sequence at

[10] Cultures were processed individually through these operations, which were timed by counting metronome signals.

the end of assay sets yielded ^{3}H-content values that were not significantly different from background values, showing that the volumes of rinsing solution used were large enough to keep levels of ^{3}H-permeant rinsed from the monolayers at sufficiently low levels that ^{3}H-uptake from the rinse media was negligible.

The cellular uptake of adenosine at time zero was approximated by exposing chilled monolayers as briefly as possible to ^{3}H-permeant solutions in the following way. After monolayer cultures in transport medium were cooled for 10 min in an ethanol-water bath at -1°, individual SK-OV-3 cultures were drained, flooded with 0.75 ml of ice-cold isotopic permeant solution (1.5 ml for other cells) and immediately immersed in 300 ml of ice-cold PBS; the monolayers were then further rinsed rapidly and consecutively in three 1-litre beakers of ice-cold PBS before assay for ^{3}H-content as described above. These assays were performed in triplicate.

Effect of NT Inhibitors on Adenosine Fluxes in Cultured Ovarian Carcinoma Cells

The effects of NBMPR, dilazep and dipyridamole on adenosine influx were evaluated in SK-OV-3 and HOC-7 cells.

Replicate monolayer cultures containing 1-2 x 10^6 cells/dish were incubated with graded concentrations of inhibitors for 15 min prior to assays of inward adenosine fluxes. Transport medium, which contained 15 μM or 20 μM ^{3}H-Ado for SK-OV-3 and HOC-7 cells, respectively, also contained designated concentrations of the inhibitors. Transport assays were performed in triplicate. Initial rates of uptake (determined from the coefficients of the first order term in least squares parabolas fitted to the time course data by polynomial regression analysis using a computer programme (HP-41C Stat Pac, Hewlett-Packard)) were expressed as percentages of control (no inhibitor) rates, and concentration-effect plots were constructed.

Competition by Nucleoside Permeants

Experiments in which proliferating RPMI 6410 cells were protected by NBMPR from a variety of cytotoxic nucleosides have shown that the facilitated diffusion transporter is tolerant of structural variations in the base portion of its substrates. Structurally diverse ribofuranosides, such as showdomycin, azomycin riboside and formycin B, are accepted as substrates by the facilitated diffusion NT system of this cell line (Cass *et al.*, 1981; Paterson *et al.*, 1979b). The broad substrate specificity of the facilitated diffusion nucleoside carrier permits the identification of a nucleoside permeation process as transporter-mediated through competitive inhibition by other transported permeants (Belt, 1983). The effects of competing purine and pyrimidine nucleosides on adenosine influx in HOC-7 cells were evaluated to determine if the process was transporter-mediated.

Inward fluxes of Ado in confluent, replicate monolayers were assayed in media containing ^{3}H-Ado (10 μM, final concentration) and graded concentrations of the non-isotopic nucleosides, uridine, inosine, deoxyadenosine, thymidine, tubercidin and adenosine. The cellular content of ^{3}H-Ado was determined as previously described and fluxes were expressed as percentages of control rates (fluxes in the absence of competing nucleosides).

Inhibitors of L-Ado Permeation

The transporter-independent route of nucleoside influx in HOC-7 cells was found to be insensitive to the classical inhibitors of facilitated diffusion NT systems. Several chemical agents, including thiol reagents, have been demonstrated to be inhibitors of various transport systems in animal cells (Belt and Noel, 1985; Ginsburg and Stein, 1987a), and the effects of several of these agents on adenosine fluxes in HOC-7 cells were evaluated. In these experiments, HOC-7 monolayers were incubated with graded concentrations of the candidate inhibitors in transport medium for 10 min prior to assay of Ado fluxes in the same media. Because pCMBS, SITS, and phloretin are light-sensitive, incubations with those agents were conducted in the dark.

E. Metabolism of Nucleosides

Replicate HOC-7 monolayers were exposed to ^{3}H-labelled permeants (formycin B, β-D-Ado, β-L-Ado) for specified intervals that were ended by the washing procedure described previously. Drained monolayers were immediately covered with 1.0 ml of ice-cold 0.4 M trichloroacetic acid and dishes were kept on ice for 30 min. Samples of the acid extracts were neutralized with equal volumes of alamine in freon (alamine 336, Freon TF; 25:75 (v/v)) (Khym, 1975). Neutralized extracts were centrifuged (10,000 x g, 3 min), and samples of the clear, aqueous layer were stored at -20° for chromatographic analysis.

Chromatographic Analysis of TCA Extracts

PEI cellulose TLC sheets were washed overnight in distilled water and air-dried just prior to use (Bols *et al.*, 1980). Ten μl portions of neutralized cell extracts were spotted on the sheets, and 1 μl portions of 20 mM solutions of carrier compounds were spotted over them. Drying at room temperature was allowed between applications. Chromatograms were developed for 90 min in water (Randerath, 1966), and carrier compounds were located under u/v light.

For the separation of inosine, adenine and hypoxanthine from adenosine, PEI cellulose-borate paper was used (Drack and Novack, 1973). PEI cellulose sheets were washed in a 10% sodium chloride solution, air-dried, and then immersed in 0.4 M triethylammonium tetraborate for 5 min. Without drying, the sheets were immersed successively in water for 1 min, in methanol for 1 min, and then air-dried overnight at room temperature. Chromatograms were developed as already described for PEI cellulose plates.

After development, 5 mm sections of chromatogram lanes were placed in scintillation vials and incubated overnight with 1 ml of 0.5 M NaOH. The extracts were neutralised with 0.1 ml of 5 M HCl, and 10 ml portions of scintillation fluid were added for radioactivity determination.

F. Octanol-Buffer Partition Coefficients

Partitioning of a solute in an octanol-water system is a frequently used approximation of that solute's ability to partition into plasma membrane lipids (Bech-Hansen *et al.*, 1975), a determinant of passive diffusion rates across the cell membrane (Naccache and Sha'afi, 1973). Partition coefficients of nucleoside permeants were determined by mixing 1 ml of *n*-1-octanol with an equal volume of water or PBS containing 150,000 to 500,000 cpm of labelled substrate (Graff *et al*, 1977). After mixing overnight on a gyratory shaker at room temperature, phases were allowed to separate and triplicate samples of 100 μl of each phase were assayed for radioactivity.

G. Countertransport

This phenomenon, also termed counterflow, provides a criterion for accepting or rejecting a carrier-mediated mechanism of permeation (Stein, 1986). Countertransport was first demonstrated by Rosenberg and Wilbrandt (1957), whose experimental design was modified slightly for this study. Replicate confluent HOC-7 monolayers were equilibrated (45 min) with 1.4 ml of 10 μM ^{3}H-formycin B in transport medium. Non-isotopic test permeants (L-ado, D-ado and formycin B) in transport medium, were added to achieve final concentrations of 1,200 μM, and at graded time intervals, assays were terminated by the washing procedure already described. Assays were performed in triplicate, and monolayers were assayed for their content of ^{3}H-formycin B. If the fluxes of a test permeant and formycin B are catalysed by the same transporter, as the test permeant flows down its concentration gradient into the cell, isotopic formycin B will be driven out of the cell against its concentration gradient until the driving (test) substrate reaches equilibrium; that level of intracellular radioactivity would be the counterflux minimum (Hofer, 1977). Intracellular radioactivity was plotted against time to find the counterflux minimum. Cells were intact after the long equilibration with formycin B at room temperature, as judged by trypan blue dye exclusion tests (98 $\pm$ 0.8% exclusion) and the absence of cell detachment.

H. Antiproliferative Effects of Nucleoside Drugs

In assaying effects of nucleoside analogues on the proliferation rates of ovarian cancer cells, replicate cultures in 60-mm petri dishes were seeded at 3 x 10^5 cells/dish in medium containing graded drug concentrations with or without 10 μM NBMPR. After 72 h at 37°, cultures were trypsinised and cells were enumerated using a Model ZF Coulter counter (Coulter Electronics, Hialeah, FLA).

Cell proliferation rates, expressed as the number of population doublings during a 72 h-growth interval, in cultures with test compounds were compared with those without additives. Assays were in triplicate, and the drugs tested were araC and 2-fluoro-9-β-D-arabinosyladenine (FaraA).

I. In Vivo Experiments

Protection

B6D2F$_1$ mice of both sexes were used in these experiments. Their ages ranged between 8 and 11 weeks. Mice in treatment groups were within a 3 g weight range, and all were of the same sex. Mice in groups of six were treated with agents dissolved in 0.15 M NaCl by ip injection in volumes proportional to 0.1 ml/10 g body weight (group average weight at the start of treatment). Control mice received injections of 0.15 M NaCl. NT inhibitors were administered in similar volumes, but by different routes. NT inhibitors were co-administered intraperitoneally with the toxicants, while inhibitors were administered prior to the toxicants through a tail vein or subcutaneously on the dorsum. The therapeutic protocol employed depended on the cytotoxic nucleoside under investigation.

Mice were weighed at the beginning of experiments and at 7-day intervals thereafter. The end-point of toxicity was mortality, which was checked daily for thirty days.

J. Chemotherapy

The Ehrlich ascites carcinoma and mouse leukaemias P388 and L1210 were maintained in $B6D2F_1$ mice by weekly ip transfer of 10^5 cells obtained from ascitic fluid. Treatment commenced 24 h (unless otherwise stated) after tumour implantation. Mice of both sexes were used, but were of the same sex for a particular set of experiments. Their ages ranged between 8 and 11 weeks and all mice were within a 3-g weight range.

Mice in groups of six were weighed at the beginning of experiments and at 5-day intervals thereafter. Mortality was checked daily and results were expressed as the ratio of the median survival time of test animals to that of control animals treated with the injection vehicle. Survival criteria for demonstration of antitumour activity were as established by the National Cancer Institute (1984). Necropsy was performed on dying mice to confirm cancer deaths as opposed to toxic drug deaths. Borderline leukaemic deaths were confirmed by spleen weights. A spleen weight of 135 mg or more was considered to signify a leukaemic death (Schabel, 1979). Weights of spleens from normal mice were about 75 mg. Cured animals were defined as those that survived for intervals more than four times longer than the median survival time of vehicle-treated mice implanted with neoplastic cells.

IV. RESULTS

A. COMBINATION CHEMOTHERAPY WITH CYTOTOXIC NUCLEOSIDES AND NT INHIBITORS

Biochemical modulation, in the context of cancer chemotherapy, is generally understood to mean the use of adjunctive agents, the use of which alone might be without therapeutic activity, to enhance the antineoplastic activity or selectivity of anticancer drugs. Such modulation is one method of attempting to overcome some of the problems associated with cancer chemotherapy, such as (i) acquired or latent tumour resistance, and (ii) host toxicity of anticancer drugs (Markman and Howell, 1987; Leyland-Jones and O'Dwyer, 1986).

Previous reports from this laboratory have shown that in treatment of experimental cancers in rodents, therapeutic indices of some intraperitoneally administered cytotoxic nucleosides, notably tubercidin and nebularine, were increased by co-administration of NBMPR-P. In an effort to maximise this therapeutic advantage, we have evaluated the use of several NT inhibitors in combination chemotherapy with different toxicants, and have investigated sc and iv routes of inhibitor administration.

B. "Two-route" Chemotherapy

A theoretical advantage of ip chemotherapy is the possibility of systemic delivery of neutralising agents concurrently with ip delivery of cytotoxic drugs in order to reduce host toxicity due to systemic drug effects, and, thereby, to increase the therapeutic index of the toxicant. This approach has been demonstrated with methotrexate-folinic acid and cisplatin-thiosulphate combinations, in treatment of ovarian carcinoma, a disease primarily confined to the peritoneal cavity (Howell *et al* ., 1981; 1982). The applicability of this tactic to the use of cytotoxic nucleoside-NT inhibitor combinations in the treatment of intraperitoneally transplanted rodent neoplasms, was investigated in the present study. Tubercidin, nebularine, 5-azacytidine, araC and 3-deazauridine were administered in combination with approximately equimolar doses of dipyridamole and dilazep, which are NT

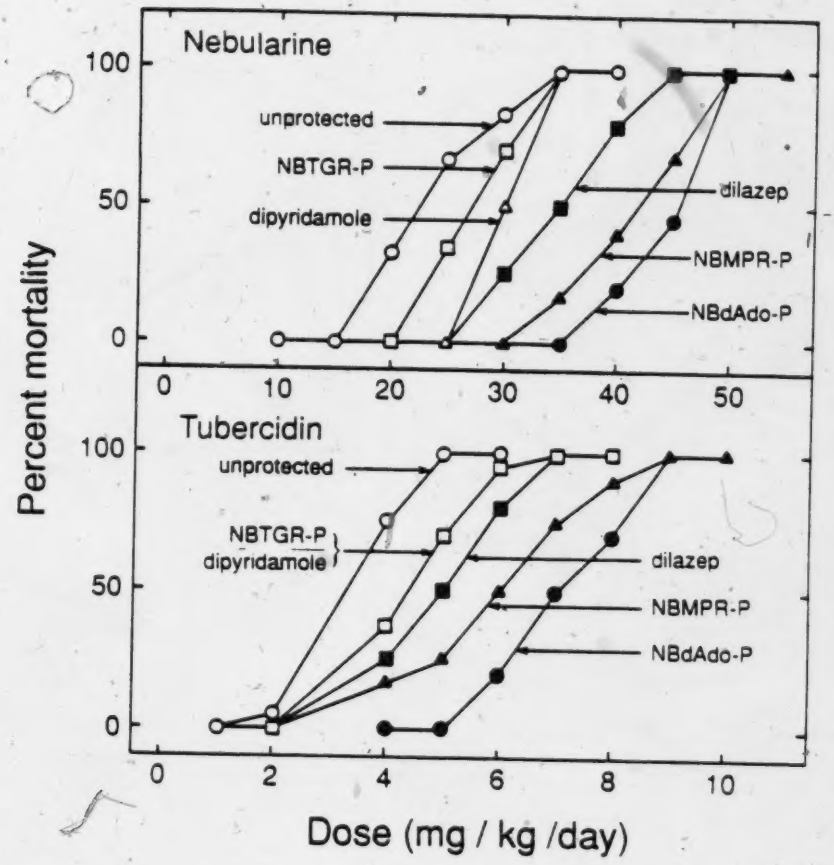


Fig. 2. Protection of mice against the lethality of nebularine and tubercidin treatment protocols by subcutaneously administered inhibitors. Male B6D2F$_1$ mice in groups of 6, were treated by the ip route with nebularine or tubercidin given at the indicated dosages at 24-h intervals; each received five (nebularine) or two (tubercidin) such treatments. The protectants, NBTGR-P (□), NBMPR-P (▲), NBdAdo-P (●), dilazep (■) and dipyridamole (△) (50mg/kg each) were administered sc 15 min before the toxicant. Deaths were recorded daily for 30 days after the first treatment.

Table 1. Protection of mice against the lethality of nebularine by NT inhibitors. Male B6D2F$_1$ mice in groups of six were treated by the ip route with graded dosages of nebularine at 24-h intervals for five days. NT inhibitors (50 mg/kg per dose) were administered subcutaneously 15 min before the toxicant. Deaths were recorded daily for 30 days. LD_{50} values were calculated from dose-mortality data as in Fig. 2 (upper panel).

Treatment group	LD_{50} (mg/kg)	Student's *t*-test
Unprotected	22.5 ± 1.5[1]	
NBTGR-P	27.0 ± 1.8	s[2]
Dipyridamole	30.0 ± 2.7	s
Dilazep	35.0 ± 1.7	s
NBMPR-P	42.0 ± 2.7	s
NBdAdo-P	45.0 ± 2.8	s

[1] Mean ± S.D. of 4 experiments.

[2] $p < 0.05$

Table 2. Protection of mice against the lethality of tubercidin by NT inhibitors. Male B6D2F$_1$ mice in groups of six received two ip injections of tubercidin at 24-h intervals. NT inhibitors (50 mg/kg per dose) were administered subcutaneously 15 min before the toxicant. Deaths were recorded daily for 30 days. LD_{50} values were obtained from dose-mortality data as in Fig. 2 (lower panel).

Treatment group	LD_{50} (mg/kg)	Student's *t*-test
Unprotected	3.3 ± 1.2[1]	
NBTGR-P	4.4 ± 1.2	ns[2]
Dipyridamole	4.4 ± 1.4	ns
Dilazep	5.0 ± 0.6	ns
NBMPR-P	6.0 ± 0.5	s
NBdAdo-P	7.0 ± 0.7	s

[1] Mean ± S.D. of 4 experiments.

[2] $p > 0.05$

inhibitors, and NBMPR-P, NBTGR-P and NBdAdo-P, which are pro-drugs of NT inhibitors (Paterson *et al.*, 1987).

Host Protection

Intravenously administered NBMPR-P, NBTGR-P and NBdAdo-P failed to protect mice against the lethality of several nucleosides tested (results not shown). In contrast, Fig. 2 illustrates the *in vivo* protection afforded $B6D2F_1$ mice against the lethality of nebularine and tubercidin by subcutaneously-administered NBMPR-P, NBTGR-P, NBdAdo-P, dipyridamole and dilazep. Mice were treated with graded dosages of tubercidin and nebularine administered by the ip route in a series of two injections (tubercidin) or five injections (nebularine), given at 24-h intervals. The protectants (50 mg/kg per dose) were administered by sc injection 15 min before the toxicants. The dosages of nebularine and tubercidin that caused 50% mortality (LD_{50}) when administered alone to mice were 22.5 mg/kg and 3.3 mg/kg, respectively. In experiments that utilised nebularine as the toxicant (Fig. 2, upper panel), each NT inhibitor significantly protected experimental mice against the lethality of nebularine (see Table 1), but a wide range of inhibitor potencies was evident. Treatment with NBMPR-P or NBdAdo-P, the most effective protectants against nebularine toxicity, doubled the tolerance of mice for this agent. Similarly, NBMPR-P and NBdAdo-P were the most potent protectants of mice against tubercidin toxicity (Fig. 2, lower panel), and treatment with either agent caused a two-fold increase in the tolerance of mice for tubercidin. Treatment with NBTGR-P, dipyridamole or dilazep did not significantly protect mice against tubercidin toxicity (Table 2). The rank order of inhibitor potencies in protection against nebularine and tubercidin lethality (LD_{50} values) was similar for the nucleoside analogues (Tables 1 and 2), but did not agree with the rank order of IC_{50} values for inhibition of Ado transport in S49 mouse lymphoma cells *in vitro* (NBMPR, NBTGR, NBdAdo and dilazep) (Paterson *et al.*, 1983b).

Table 3a. Treatment of mice bearing ip implants of the Erhlich ascites carcinoma (EAC) with ip tubercidin and sc NBMPR-P. Male B6D2F$_1$ mice in groups of six received 10^6 EAC cells intraperitoneally and treatment (qd, days 1-4, dosages specified below) was started 24 h later. The protectant, NBMPR-P, was administered subcutaneously 15 min before the toxic agent. The median survival time of untreated mice was 13 days. Deaths were recorded daily.

Treatment					
Agent	Dose (mg/kg/day)	Median survival (days)	T/C (%)	ILS[1] (%)	Cures (%)
Tubercidin	1	27	208	108	30
	2	5.5	50	lethal	0
Tubercidin + NBMPR-P	2 + 50	46	354	254	65
NBMPR-P	50	13.5	104	4	0

[1] ILS (Increase in life span): The difference between median survival times of treated and untreated (control) animals that died expressed as a percentage of the control median survival time (13 days).

Table 3b. Treatment of mice implanted with leukaemia P388 cells with ip tubercidin and sc NBMPR-P. Male B6D2F$_1$ mice in groups of six received 10^6 leukaemic cells intraperitoneally and treatment (qd, days 1-4, dosages specified below) was started 24 h later. The protectant, NBMPR-P, was administered subcutaneously 15 min before the toxic agent. The median survival time of untreated mice was 9.5 days. Deaths were recorded daily.

Treatment					
Agent	Dose (mg/kg/day)	Median survival (days)	T/C (%)	ILS[1] (%)	Cures (%)
Tubercidin	1	13.5	142	42	0
	2	8.5	89	toxic	0
Tubercidin + NBMPR-P	2 + 50	17.5	184	84[2]	0
NBMPR-P	50	9.5	105	5	0

[1] Increase in life span (see Table 3a).

[2] Tubercidin lethality was not evident in NBMPR-P treated mice. Those mice died from leukaemia.

Table 3c. Treatment of mice implanted with leukaemia L1210 cells with ip tubercidin and sc NBMPR-P. Male B6D2F$_1$ mice in groups of six received 10^6 leukaemic cells intraperitoneally and treatment (qd, days 1-9, dosages specified below) was started 24 h later. The protectant, NBMPR-P, was administered subcutaneously 15 min before the toxic agent. The median survival time of untreated mice was 9 days. Deaths were recorded daily.

Treatment					
Agent	Dose (mg/kg/day)	Median survival (days)	T/C (%)	ILS[1] (%)	Cures (%)
Tubercidin	0.5	9	100	0	0
	1	8	9	toxic	0
Tubercidin NBMPR-P	1 50	13	144	44[2]	0
NBMPR-P	50	9	100	0	0

[1] Increase in life span (see Table 3a)

[2] Leukaemic deaths (see Table 3a).

Subcutaneous treatment of mice with 50 mg/kg each of the protectants investigated in the foregoing experiments, failed to protect those mice against the toxicity of ip dosages of 3-deazauridine, 5-azacytidine and araC (results not shown), indicating that the host-protective effect of the NT inhibitors differed with the nucleoside analogue employed as the toxicant.

Chemotherapy

It is seen in the results of Fig. 2 (and Tables 1 and 2) that significant protection was afforded mice against the lethality of tubercidin and nebularine by NT inhibitors. The experiments summarised in Tables 3a, 3b, and 3c were concerned with the ip tubercidin-sc NBMPR-P therapy of mice bearing 10^6 intraperitoneally implanted cells of the following neoplasms: the Erhlich ascites carcinoma (EAC), leukaemia P388, or leukaemia L1210 cells. The data of Table 3a demonstrated that single-agent therapy with tubercidin in a tolerated regimen (4 doses of 1 mg/kg at 24-h intervals) was effective against EAC cells, in that the median survival time of mice that succumbed to the neoplasm was doubled, and 30% of the treated were long-term survivors (cures). Tubercidin toxicity was apparent in the reduced survival times of mice treated with 2 mg/kg tubercidin, as compared to untreated controls. This otherwise lethal dose of tubercidin when administered in combination with 50 mg/kg NBMPR-P was not toxic to the experimental mice, and led to 65% cures and a four-fold increase in the life span of mice that died of their neoplasms. These results were significantly superior to those achieved by single-agent therapy with the tolerated tubercidin dose of 1 mg/kg ($p < 0.05$). NBMPR-P, administered alone, had no anti-neoplastic effect.

The mouse leukaemias P388 and L1210 were less sensitive than the Ehrlich ascites carcinoma to therapy on the qd x 4 treatment schedule with tubercidin alone, or with the tubercidin-NBMPR-P combinations, in that among the treated, leukaemic mice no long-term survivors emerged. In mice with intraperitoneally-implanted leukaemia P388 cells, treatment with the tubercidin-NBMPR-P combination increased median survival time by 84% relative to untreated controls (Table 3b). This increase in median survival time was not significantly

Table 4a. Treatment of mice implanted with leukaemia P388 cells with ip nebularine and sc NBMPR-P. Female B6D2F$_1$ mice in groups of six were inoculated with 10^6 P388 leukaemia cells by ip injection. Treatment with ip nebularine (qd, days 1-5, dosages specified below) started 24 h after implantation of neoplastic cells. The protectant, NBMPR-P, was administered subcutaneously 15 min before the cytotoxic agent. The median survival time of untreated (control) mice was 9 days. Deaths were recorded daily. Leukaemic deaths were confirmed by spleen weights.

Treatment					
Agent	Dose (mg/kg/day)	Median survival (days)	T/C (%)	ILS[1] (%)	Cures (%)
Nebularine	15	13	144	44	0
	25	13[2]	144	44	0
Nebularine NBMPR-P	25 50	19	210	110[3]	0
NBMPR-P	50	9	100	0	0

[1] Increase in life span (see Table 3a)

[2] 50% of mice died from drug toxicity.

[3] Nebularine toxicity was not evident in NBMPR-P treated mice. Those mice died from leukaemia.

Table 4b. Treatment of L1210 leukaemia-bearing mice with ip nebularine and sc NBMPR-P. Female B6D2F$_1$ mice in groups of six were inoculated with 10^6 L1210 leukaemia cells by ip injection. Treatment with ip nebularine (qd, days 1-9, dosages specified below) started 24 h after implantation of neoplastic cells. The protectant, NBMPR-P, was administered subcutaneously 15 min before the cytotoxic agent. The median survival time of untreated mice was 8 days. Deaths were recorded daily. Leukaemic deaths were confirmed by spleen weights.

Treatment					
Agent	Dose (mg/kg/day)	Median survival (days)	T/C (%)	ILS[1] (%)	Cures (%)
Nebularine	10	10.5	131	31	0
	20	7	88	toxic	0
Nebularine NBMPR-P	20 50	15	188	88[2]	0
NBMPR-P	50	8.5	106	6	0

[1] Increase in life span (see Table 3a)

[2] Leukaemic deaths (see Table 4a).

greater than the increase achieved by tolerated dosages of tubercidin administered alone (42%) ($p > 0.05$). In contrast, the tubercidin-NBMPR-P combination significantly prolonged the lives of L1210-bearing mice ($p < 0.05$) relative to mice receiving single-agent therapy with tolerated dosages of tubercidin. The median survival time of the former group of mice was increased by 44% relative to that of untreated controls, while the median survival time of the latter group was similar to that of untreated controls (Table 3c). Tables 4a and 4b present data from experiments in which mice implanted intraperitoneally with 10^6 leukaemia P388 and L1210 cells were treated with five doses and nine doses of ip nebularine, respectively, at 24-h intervals with or without sc NBMPR-P (50 mg/kg). Both leukaemias were slightly responsive to the nebularine treatment protocols. Tolerated dosages of nebularine administered alone, increased the median survival of L1210 and P388 leukaemia-bearing mice by 44% (Table 4a) and 31% (Table 4b), respectively, relative to untreated controls. Combination of subcutaneously administered NBMPR-P with potentially lethal doses of nebularine, led to 110% and 88% increases in life span of leukaemia P388 and L1210-bearing mice, respectively, relative to untreated (control) mice. These results were significantly superior to those from groups treated with nebularine alone ($p < 0.05$).

With the exception of the tubercidin-NBMPR-P combination in the treatment of P388 leukaemia (Table 3b), results of treatment with the foregoing inhibitor-toxicant combinations in the "two-route" chemotherapy approach represent moderate to significant antineoplastic activity by NCI(US) criteria (NIH Publication No. 84-2635, 1984). For intraperitoneally-implanted L1210 leukaemia in $B6D2F_1$ mice, increases in survival representing T/C values $\geq$ 125% resulting from therapy with an investigational drug are considered necessary to demonstrate moderate activity of the drug. Reproducible T/C values $\geq$ 150% indicate significant anti-neoplastic activity by NCI(US) criteria. For intraperitoneally-implanted P388 leukaemia cells in $B6D2F_1$ mice, increases in survival representing T/C values $\geq$ 120% and $\geq$ 175% resulting from therapy with an investigational drug demonstrate moderate and significant antineoplastic activity of the drug (NIH Publication No. 84-2635, 1984).

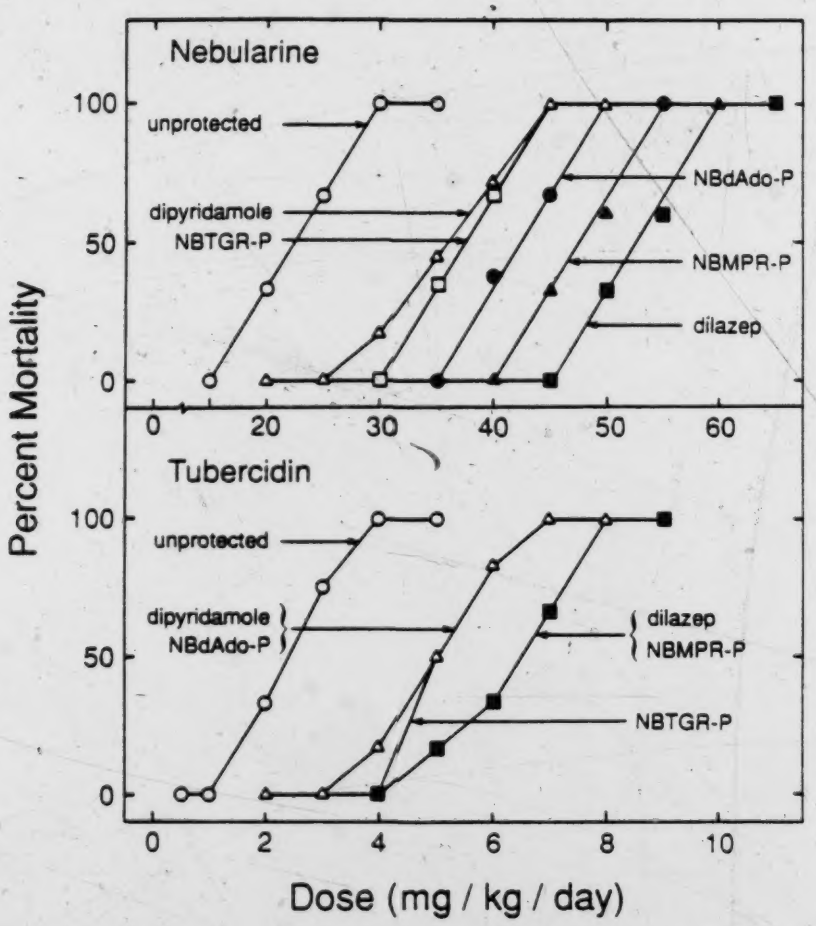


Fig. 3. Protection of mice against the toxicity of nebularine and tubercidin treatment protocols by intraperitoneally administered NT inhibitors. Male B6D2F$_1$ mice received 5 intraperitoneal injections of nebularine and 4 of tubercidin at 24-h intervals. The NT inhibitors (50 mg/kg per dose) NBTGR-P(□), NBMPR-P(▲), NBdAdo-P(●), dilazep (■) and dipyridamole(△) were co-administered intraperitoneally. Deaths were recorded daily for 30 days after the initial treatment.

Table 5. Protection of mice against the lethality of tubercidin by NT inhibitors.

Male B6D2F$_1$ mice in groups of six received four injections of graded ip dosages of tubercidin, co-administered with 50 mg/kg of the inhibitors at 24-h intervals. Deaths were recorded daily for 30 days. LD_{50} values were calculated from dose-mortality data depicted in Fig. 3 (lower panel).

Treatment group	LD_{50} (mg/kg)	Student's *t*-test
Unprotected	2.4 ± 0.5[1]	
Dipyridamole	5.0 ± 1.2	s[2]
NBdAdo-P	5.0 ± 1.0	s
NBTGR-P	5.0 ± 0.7	s
Dilazep	6.8 ± 1.0	s
NBMPR-P	6.8 ± 0.5	s

[1] Mean ± S.D. of 4 experiments.

[2] $p < 0.05$

Table 6. Protection of mice against the lethality of nebularine by NT inhibitors.

Male B6D2F$_1$ mice in groups of six received five injections of graded ip doses of nebularine, administered with 50 mg/kg of inhibitors at 24-h intervals. Deaths were recorded daily for 30 days. LD_{50} values were derived from dose-mortality data depicted in Fig. 3 (upper panel).

Treatment group	LD_{50} (mg/kg)	Student's *t*-test
Unprotected	22.5 ± 2.3[1]	
Dipyridamole	36.0 ± 2.3	s
NBTGR-P	37.5 ± 1.6	s
NBdAdo-P	42.5 ± 2.5	s
NBMPR-P	47.5 ± 4.3	s
Dilazep	52.5 ± 2.8	s

[1] Mean ± S.D. of 4 experiments.

C. Intraperitoneal Administration of Inhibitor and Toxicant

The ip co-administration of NT inhibitors and cytotoxic nucleosides has been used in chemotherapy of experimental tumours (Jakobs and Paterson, 1984; Lynch *et al.*, 1981a,b; Paterson *et al.*, 1979a; 1983a) and parasitic diseases (El Kouni *et al.*, 1983,1987; Ogbunude and Ikediobi,1982; Gati *et al.*, 1987). In most of these studies, the use of NT inhibitors was limited to NBMPR-P. The potency of other NT inhibitors co-administered by the ip route different nucleoside toxicants, was investigated in the present work.

Host Protection

The toxicities of intraperitoneally-administered dosages of nebularine (qd x 5) and tubercidin (qd x 4) are illustrated in Fig. 3. The ip co-administration of the NT inhibitors, NBTGR-P, NBMPR-P, NBdAdo-P, dilazep or dipyridamole (50 mg/kg per dose) increased the tolerance of mice to the toxic nucleoside analogues. Such tolerance represented two- to three-fold increases in LD_{50} values found with the tubercidin-dilazep and tubercidin -NBMPR-P combinations (Table 5). Two-fold increases in LD_{50} values were found with the nebularine-dilazep and nebularine-NBMPR-P combinations (Table 6). As well, the other NT inhibitors evaluated significantly increased the tolerance of mice to the toxicity of tubercidin and nebularine (Tables 5 and 6). The rank order of inhibitor potencies in these studies was different from that observed for sc inhibitor administration (Fig. 2), indicating that one determinant of *in vivo* NT inhibitor effectiveness is the route of administration.

Fig. 4 summarises the toxicity of intraperitoneally administered araC (q-8h x 12) and 5-azacytidine (qd x 5) to mice, and shows that ip co-administration of NT inhibitors increased to a minor extent the tolerance of mice to the toxicity of araC. This increase was not significant for dilazep, and was less than two-fold for dipyridamole, NBMPR-P and NBdAdo-P (Table 7), indicating that the NT inhibitors protected mice less well against the

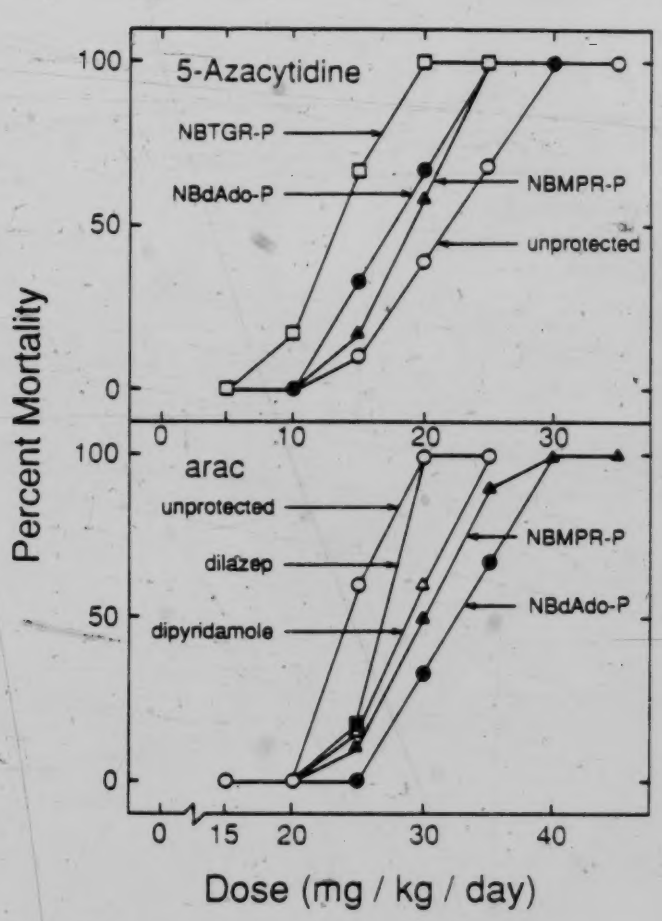


Fig. 4. Administration of cytotoxic nucleosides with NT inhibitors by the ip route. Female B6D2F$_1$ mice received 4 injections of 5-azacytidine at 24-h intervals, or twelve injections of araC at 8-h intervals. The NT inhibitors, NBTGR-P(□), NBMPR-P(▲), NBdAdo-P (●), dilazep(■) or dipyridamole(△), were administered prior to the toxicants at 50 mg/kg per dose. Deaths were recorded daily for 30 days after the initial treatment.

Table 7. Protection of mice against the lethality of araC by NT inhibitors. Female B6D2F$_1$ mice in groups of six received 12 injections of graded araC doses by the ip route at 8-h intervals. NT inhibitors (50 mg/kg per dose) were administered with the toxicant. Deaths were recorded daily for 30 days. LD_{50} values were obtained from dose-mortality data depicted in Fig. 4 (lower panel).

Treatment group	LD_{50} (mg/kg)	Student's *t*-test
Unprotected	24.2 ± 2.4[1]	
Dilazep	26.8 ± 1.2	ns[2]
Dipyridamole	28.9 ± 1.8	s[3]
NBMPR-P	30.0 ± 2.9	s
NBdAdo-P	32.4 ± 2.4	s

[1] Mean ± S.D. of 4 experiments.

[2] $p > 0.05$

[3] $p < 0.05$

Table 8. Enhancement of the lethality of 5-azacytidine by NT inhibitors. Female B6D2F$_1$ mice in groups of six received four injections of graded 5-azacytidine doses by the ip route at 24-h intervals. NT inhibitors (50 mg/kg per dose) were administered with the toxicant. Deaths were recorded daily for 30 days. LD_{50} values were obtained from dose-mortality data depicted in Fig. 4 (upper panel).

Treatment group	LD_{50} (mg/kg)	Student's *t*-test
Unprotected	22.0 ± 2.0[1]	
NBMPR-P	19.3 ± 1.5	ns
NBdAdo-P	17.3 ± 2.5	ns
NBTGR-P	13.2 ± 2.0	s

[1] Mean ± S.D. of 3 experiments.

toxicity of araC than against that of tubercidin or nebularine. Intraperitoneal co-administration of NBTGR-P enhanced the lethality of 5-azacytidine to mice. NBMPR-P and NBdAdo-P had no significant effect on 5-azacytidine toxicity to mice (Table 8). The enhancement of toxicity observed in this study has been previously described for certain other nucleoside-NT inhibitor combinations, such as the pyrazofurin-NBMPR-P and 5-fluorouridine-dilazep combinations (Paterson *et al.*, 1983). These results suggest that *in vivo* protection against the lethality of cytotoxic nucleoside analogues by NT inhibitors is not a general phenomenon, but depends on the properties of individual inhibitors and toxicants.

D. NUCLEOSIDE TRANSPORT IN HUMAN OVARIAN CARCINOMA CELLS

In an effort to apply the foregoing chemotherapeutic strategy to the treatment of human neoplastic disease, nucleoside permeation characteristics of ovarian carcinoma cells were explored. Because of the size of the population served by the Cross Cancer Institute, samples of ascitic fluid from ovarian cancer patients were not regularly available. Secondly, such samples contained non-malignant mesothelial cells that were difficult to separate from neoplastic cells. As well, neoplastic cell yield from ascitic fluid samples varied widely with sample batch. For these practical reasons, cultured human ovarian carcinoma cells were used for these studies.

E. SK-OV-3 Cells

This cell line was established in culture in 1973 from the ascitic fluid of a 64-year old patient with a primary serous cystadenocarcinoma of the ovary, who had been treated with thiotepa prior to the establishment of the ovarian cells in culture (Fogh and Trempe, 1975). The tumour has been maintained in McCoy's 5a medium with 15% foetal bovine serum (Fogh *et al.*, 1977).

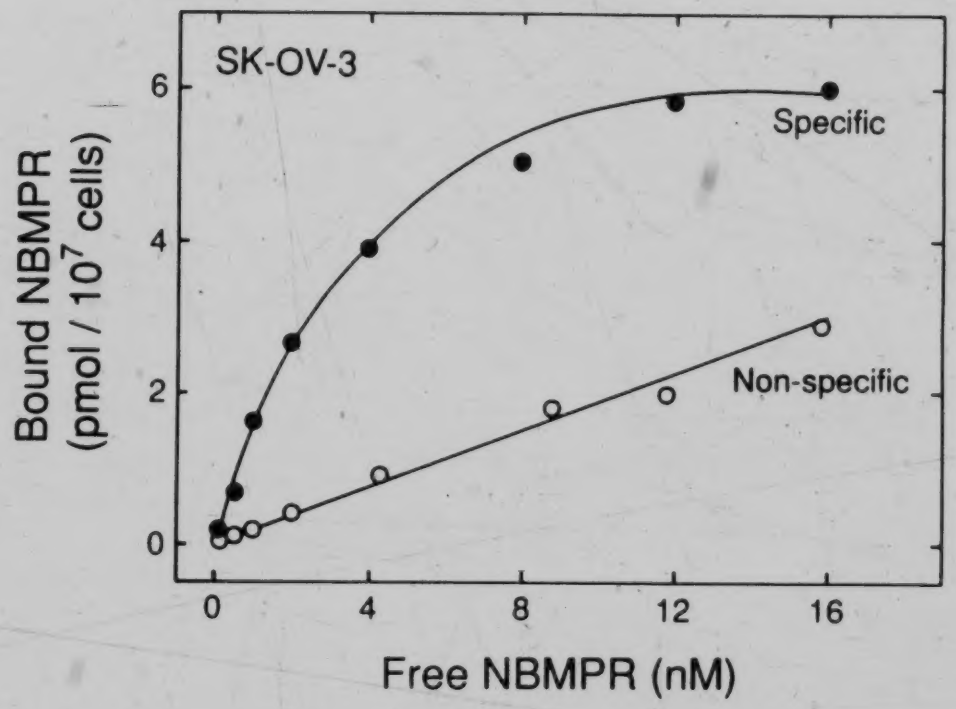


Fig. 5. Saturable, site-specific binding of NBMPR by SK-OV-3 ovarian carcinoma cells. Replicate monolayer cultures were exposed for 20 min at 22° to PBS containing graded concentrations of ^{3}H-NBMPR with and without 10 μM non-isotopic NBMPR. The ^{3}H-content of medium and cells was determined as described in Materials and Methods. Site-specific binding of ^{3}H-NBMPR (●) was determined as the difference between (i) the cell content of ^{3}H-NBMPR in the absence of NBMPR , and (ii) that in the presence of 10 μM non-isotopic NBMPR (○). Means of triplicate assays are plotted for an experiment typical of 6 similar experiments.

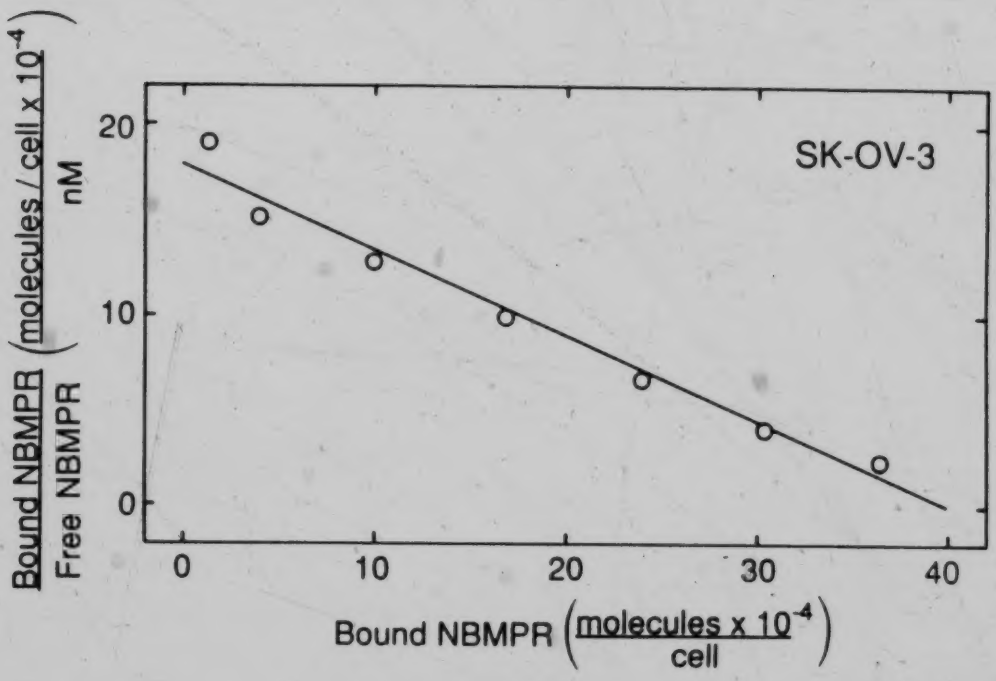


Fig. 6. Site-specific binding of NBMPR to SK-OV-3 cells. Mass law analysis by the method of Scatchard (Edsall and Wyman, 1958) of data from Fig. 5 for the site-specific binding of NBMPR to SK-OV-3 cells was performed by the Ligand microcomputer programme (Munson and Rodbard, 1980). The data shown are means of triplicate assays and are typical of 6 similar experiments for which the mean binding constants (± S.E.) were; K_D, 2.2 ± 0.3 nM, and B_{max}, 4.0 ± 0.6 x 10^5 molecules/cell.

Presence of High-affinity NBMPR Binding Sites on SK-OV-3 Cells

The first step in characterising nucleoside transport in SK-OV-3 cells involved the evaluation of site-specific binding of NBMPR to these cells by equilibrating replicate monolayers with graded concentrations of ^{3}H-NBMPR in the presence and absence of 10 μM NBMPR. Differences between the cell content of ^{3}H-NBMPR at equilibrium in the presence and absence of non-isotopic NBMPR is defined as site-bound or specifically bound NBMPR. Fig. 5 illustrates the saturability of site-specific binding of NBMPR to SK-OV-3 cells. Fig. 6 illustrates mass law analysis of the binding data using the Ligand microcomputer programme (Munson and Rodbard, 1980), and indicates that B_{max}, the maximum number of NBMPR-specific binding sites and K_D, the dissociation constant for site-bound NBMPR were $4.0 \pm 0.6 \times 10^{5}$ sites/cell and 2.2 ± 0.4 nM, respectively. The linearity of the Fig. 6 plot indicates the presence of a single class of high-affinity NBMPR binding sites on SK-OV-3 cells. The binding constants obtained are similar to reported values for other lines of cultured human neoplastic cells (Chen *et al.*, 1986; Jamieson *et al.*, 1989).

Inhibition of Adenosine Transport

The sensitivity to NT inhibitors of inward fluxes of Ado in SK-OV-3 cells was evaluated in experiments that measured time courses of ^{3}H-Ado influx over brief intervals (0-15sec). Replicate monolayer cultures were incubated in transport medium in the presence or absence of graded concentrations of NBMPR, dilazep and dipyridamole. Intervals of ^{3}H-Ado influx were started by flooding monolayers with permeant-containing medium and ended by (i) aspirating medium, and (ii) rapidly immersing dishes in ice-cold PBS. Initial rates of Ado uptake (that is, Ado fluxes) were determined as slopes of the uptake time courses, which were calculated as the coefficients the first order term in least squares parabolas fitted to the influx data. Fluxes measured in the presence of inhibitors were plotted as percentages of control fluxes (absence of inhibitors); examples of such plots (concentration-effect curves) are shown in Fig. 7. Adenosine permeation in these cells is seen to have at least two components: (i) a component of high NBMPR sensitivity

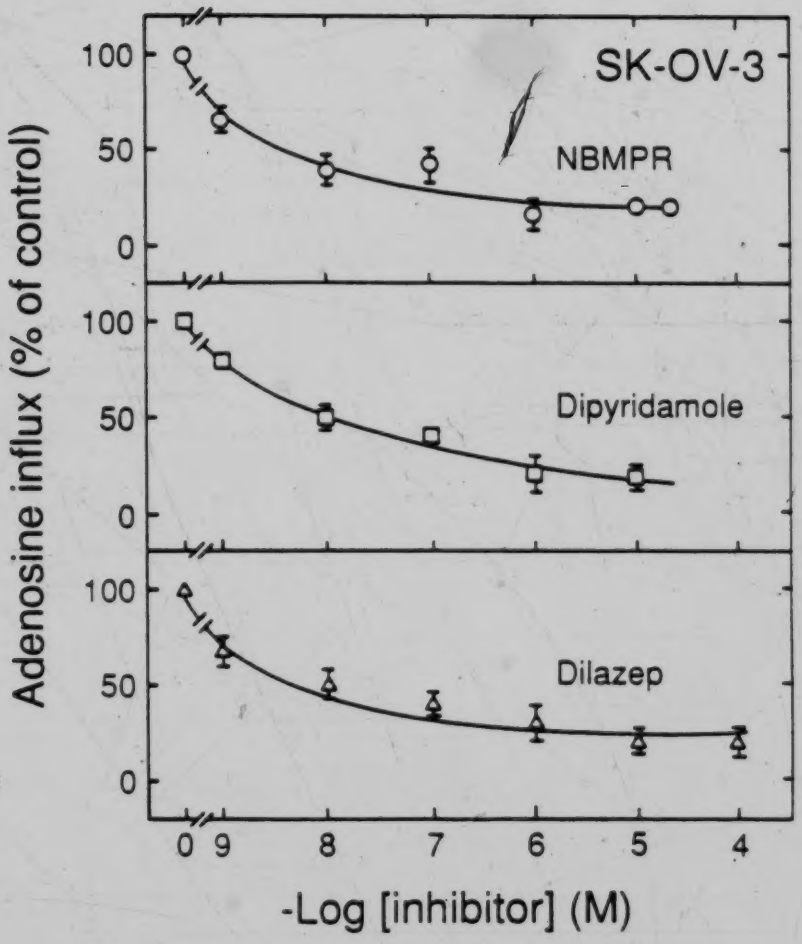


Fig. 7. Inhibition of Ado fluxes in SK-OV-3 cells. Replicate monolayer cultures were exposed at 22° for 20 min to graded concentrations of the NT inhibitors, NBMPR, dipyridamole or dilazep. To construct time courses, intervals of Ado uptake were started by adding to culture dishes transport medium containing ^{3}H-Ado (20 μM) and graded concentrations of the inhibitors and ended by immersing monolayers in ice-cold PBS. Assays were conducted in triplicate. Ado fluxes were determined from the coefficients of the first order term in least squares parabolas fitted to the time course data, and were expressed as percentages of fluxes in the absence of NT inhibitors (6.9 ± 0.8 pmol/10^{6} cells/sec, 100%). The data shown are means ± S.E. from 4 similar experiments.

(IC_{50} < 10 nM), and (ñ) a component of low NBMPR sensitivity. The concentration-effect relationships of the three inhibitors are similar, suggesting similar interactions of NT inhibitors with the nucleoside transport polypeptide(s) of this cell type.

Kinetics of Adenosine Transport

Nucleoside transport in animal cells is a rapid process and rapid sampling methods are required in order to measure nucleoside fluxes (Paterson *et al.*, 1981a,b ; Wohlhueter *et al.*, 1978). In this study, Ado fluxes were measured as initial rates of nucleoside uptake determined from early time courses of cellular influx, as indicated in earlier sections. The cellular uptake of ^{3}H-Ado at graded concentrations was measured at intervals between 0 and 15 sec to construct such time courses (Fig. 8). In the measurement of time-zero values for ^{3}H-Ado uptake, monolayer cultures in transport medium were cooled for 10 min in a water-bath at 0°C. After aspirating the medium, the monolayers were individually flooded with ice-cold ^{3}H-Ado solutions in transport medium and dishes were at once immersed in 300 ml of ice-cold PBS. As shown in Fig. 9, the time-zero values for the Ado content of the monolayers were proportional to permeant concentration, indicating that this parameter was determined by adsorption and other physicochemical properties of the system.[11]

Adenosine fluxes in SK-OV-3 cells are plotted against Ado concentrations in Fig. 10. This plot shows rate saturability and, therefore, demonstrates that Ado permeation in this system is transporter-mediated. Analysis of the Fig. 10 velocity-concentration data by a Hanes-Woolf plot (Hanes, 1932; Woolf, 1932) yielded the following kinetic constants: K_m, 39 ± 6 μM; V_{max}, 7.6 ± 0.9 pmol / 10^6 cells/ sec (Fig. 11). These values agree with those obtained in other cultured human cell lines (Harley *et al.*, 1982) and these data demonstrate the presence of a carrier-mediated NT system in SK-OV-3 cells.

[11] Radioactivity associated with blank dishes (no cells attached) that were processed through similar operations was minimal and not proportional to permeant concentration.

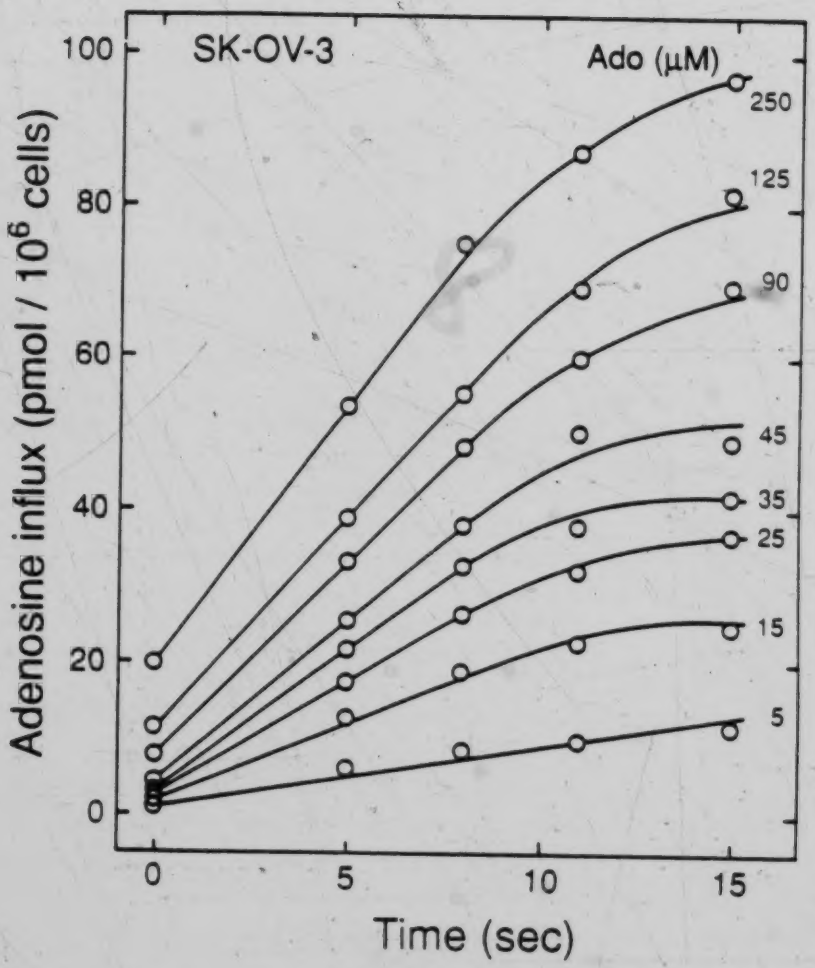


Fig. 8. Time courses of Ado influx in SK-OV-3 cells. Intervals of Ado influx in replicate monolayer cultures were started by addition of transport medium containing ^{3}H-Ado to culture dishes and ended by immersing the monolayers in ice-cold PBS. Intracellular permeant was quantitated by liquid scintillation counting after cell solubilisation with 5% Triton X-100. The data shown are means of triplicate assays and are typical of 3 similar experiments.

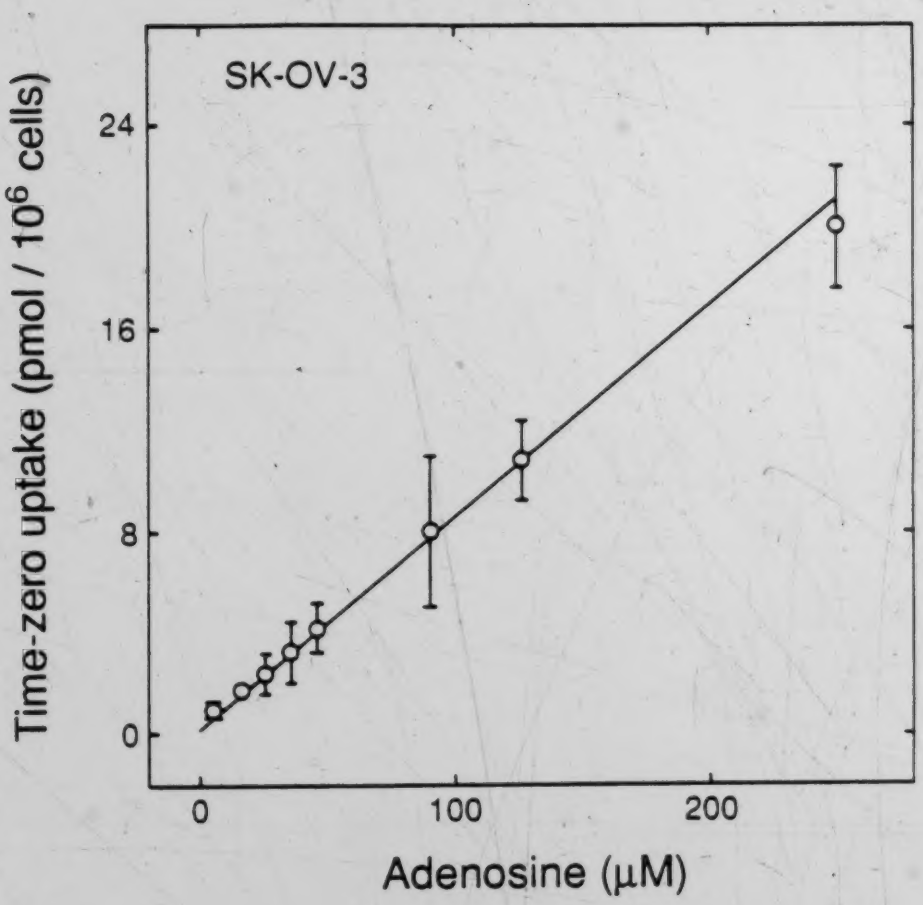


Fig. 9. Dependence of time-zero Ado uptake by SK-OV-3 monolayers on permeant concentration. Monolayer cultures in transport medium were cooled in a water bath at -1°C. After aspirating transport medium, monolayers were flooded with graded concentrations of ^{3}H-Ado in ice-cold transport medium and rapidly immersed in 300 ml of ice-cold PBS. Cell-associated permeant was quantitated as described in Fig. 8. Data shown are means ± S.E. from 3 similar experiments in each of which assays were conducted in triplicate.

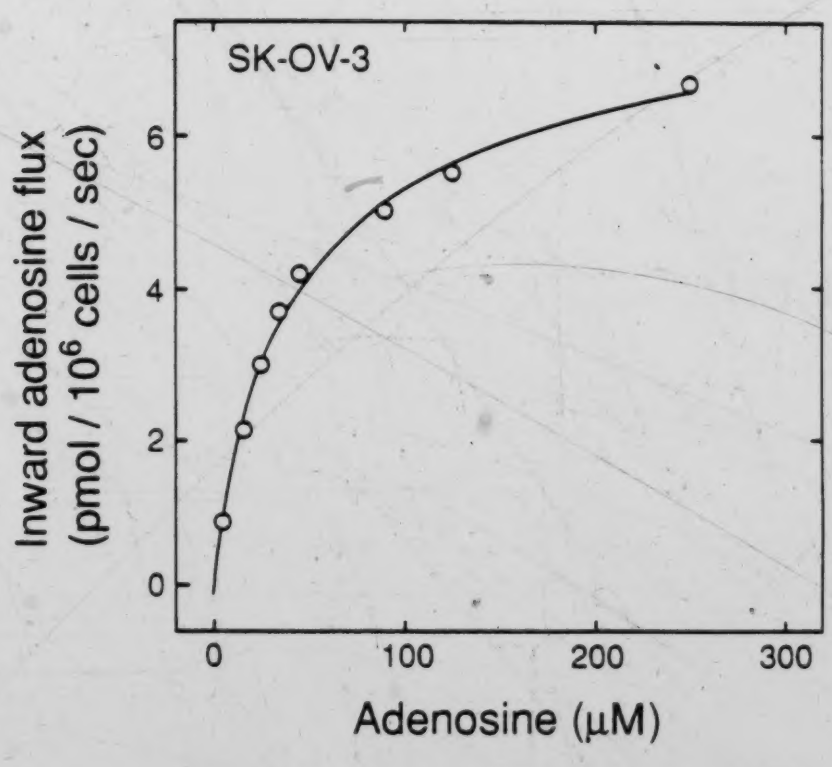


Fig. 10. Saturability of Ado fluxes in SK-OV-3 cells. Adenosine fluxes in monolayer cells were determined as initial rates from the time course data in Fig. 8 by the curve fitting method as described in Materials and Methods. The data shown are means of triplicate assays and are typical of 3 similar experiments.

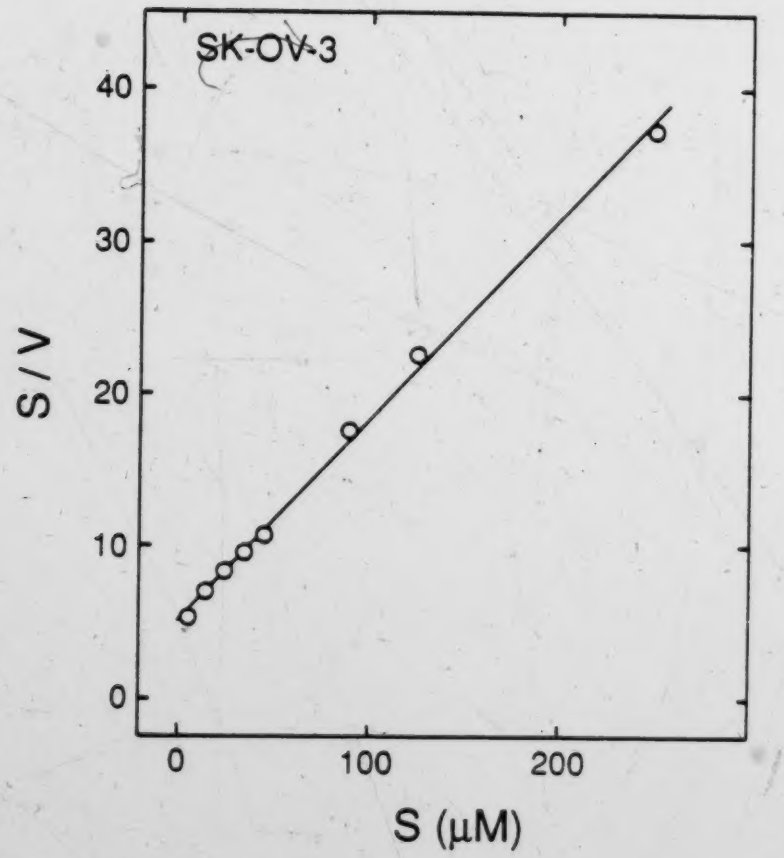


Fig. 11. Flux-concentration relationships in permeation of Ado in SK-OV-3 monolayer cells. Flux-concentration data from Fig. 10 were analysed by the Hanes-Woolf method (Hanes, 1932; Woolf, 1932). These data are typical of 3 similar experiments; averaged kinetic constants ± S.E. from the 3 experiments were K_m, 39 ± 6 μM; and V_{max}, 7.6 ± 0.9 pmoles/10^6/sec, respectively.

F. HOC-7 Cells

This cell line was derived in 1980 from the ascitic fluid of a patient with a serous well-differentiated stage III adenocarcinoma of the ovary. Cells were established in α-MEM medium supplemented with 10% foetal calf serum and have been maintained in this medium

Site-specific Binding of NBMPR to HOC-7 cells

High affinity NBMPR binding sites on HOC-7 eells were enumerated by equilibrating cells with graded concentrations of ^{3}H-NBMPR, in the presence and absence of excess non-isotopic NBMPR. Differences between the equilibration cell content of ^{3}H-NBMPR in the presence and absence of non-isotopic NBMPR were the basis of measurements of specifically bound NBMPR.

Fig. 12 demonstrates the association of graded concentrations of ^{3}H-NBMPR with HOC-7 cells by binding in a saturable fashion to specific sites and by binding non-specifically. Mass law analysis (Fig. 13) of these data by the method of Scatchard, using the Ligand microcomputer programme (Munson and Rodbard, 1980), yielded the following binding constants: B_{max}, 9.0 ± 0.9 x 10^{5} sites/cell; K_D, 2.9 ± 0.4 nm, respectively. The linearity of the Scatchard plot indicates that a single class of sites bind NBMPR.

Inhibition of Adenosine Transport

As in the experiments with SK-OV-3 cells described earlier, the sensitivity of inward Ado fluxes in HOC-7 cells to NT inhibitors was evaluated by employing brief intervals of cell exposure to Ado. Time courses based on short intervals of cell exposure to permeant in the

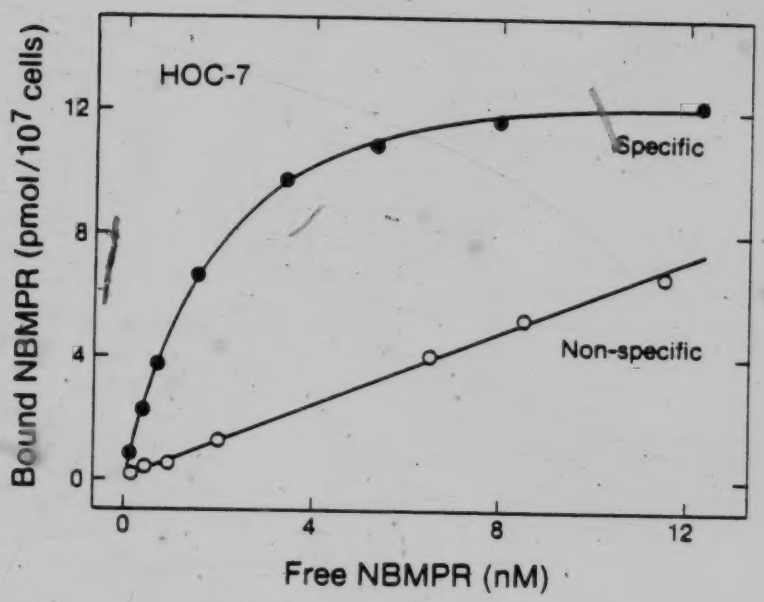


Fig. 12. Site-specific binding of NBMPR to HOC-7 cells. Replicate monolayer cultures were exposed for 20 min at 22° to PBS containing graded concentrations of ^{3}H-NBMPR with and without 10 μM non-isotopic NBMPR. The ^{3}H-content of medium and cells were determined as described in Materials and Methods. Site-specific binding of ^{3}H-NBMPR (●) was determined as the difference between the cell content of ^{3}H-NBMPR in the absence of NBMPR and that in the presence of 10 μM non-isotopic NBMPR (○). The data shown, means of triplicate assays, are typical of 6 similar experiments.

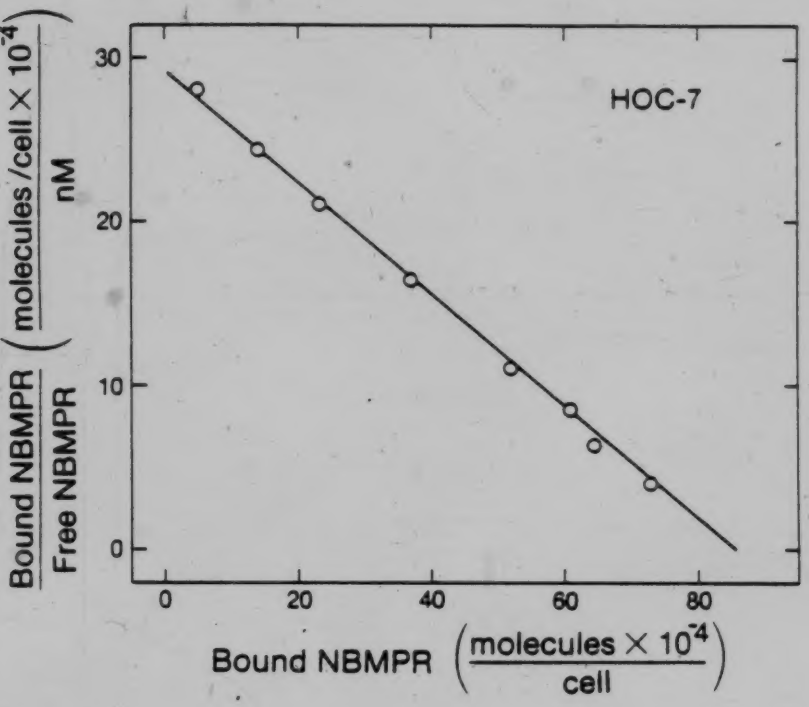


Fig. 13. Site-specific binding of NBMPR to HOC-7 cells. Mass law analysis by the method of Scatchard of data from Fig. 12 for the site-specific binding of NBMPR to HOC-7 cells was performed using the Ligand microcomputer programme. The data shown are means of triplicate assays and are typical of 6 similar experiments for which the mean binding constants (± S.E.) were K_D, 2.9 ± 0.4 nM; and B_{max}, 8.6 ± 0.9 x 10^5 molecules/cell.

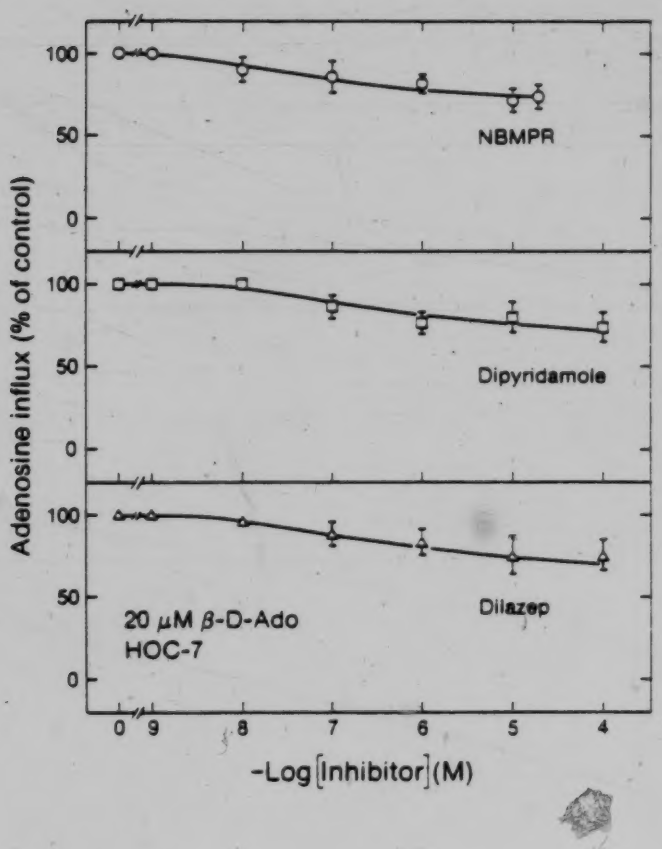


Fig. 14. Partial inhibition of inward Ado fluxes by NT inhibitors. Replicate HOC-7 monolayers were incubated for 15 min with graded concentrations of the NT inhibitors, NBMPR, dipyridamole and dilazep. Intervals of Ado influx were started by addition of transport medium containing ^{3}H-Ado and graded concentrations of the inhibitors, and were ended by immersion of monolayers in ice-cold PBS. Ado fluxes, determined as described in Fig. 7, were expressed as percentages of fluxes in the absence of inhibitors (5.8 ± 0.6 pmol/10^{6} cells/sec, 100%). The data shown are means ± S.E. from 4 similar experiments.

presence and absence of graded concentrations of NBMPR are needed to define initial rates of permeant uptake, which, by definition, measure unidirectional, inward fluxes of that permeant (Paterson and Cass, 1986; Plagemann *et al.*, 1988). Fluxes of ^{3}H-Ado (20 μM) in HOC-7 cells exposed to the inhibitors, NBMPR, dipyridamole or dilazep for 15 min prior to transport assays, were measured as described in Fig. 7. Such fluxes are plotted in Fig. 14 as percentages of control fluxes measured in the absence of inhibitors (5.8 $\pm$ 0.6 pmol/10^{6} cells/sec[12], 100%). Those fluxes were of low sensitivity to the NT inhibitors, with about 70% of Ado permeation remaining in the presence of 20 μM inhibitor. The pattern of Ado permeation inhibition was similar for all three NT inhibitors.

These results differ from those obtained in experiments with several types of cultured animal cells in which the inhibition of nucleoside transport by dipyridamole appeared to be different from inhibition by NBMPR. For instance, Belt and Noel (1985) found that in Walker 256 cells, dipyridamole (10 μM) blocked NBMPR-insensitive fluxes of uridine (100 μM). As well, inward fluxes of araC (1 μM) in HL-60 cells were only partially inhibited by 10 μM NBMPR, but were blocked virtually completely by 10 μM dipyridamole (Kubota *et al.*, 1988). In contrast, the inhibitory effects of NBMPR and dipyridamole on Ado permeation in HOC-7 cells were similar.

Kinetics of Adenosine Transport

The relationship of inward Ado fluxes in HOC-7 cells to Ado concentration was explored in the experiment of Fig. 15, which presents time courses for Ado influx at graded concentrations of ^{3}H-Ado. Time-zero values for the Ado content of monolayers were obtained as already described for SK-OV-3 cells. Those values were proportional to Ado concentration, indicating that they were determined by physicochemical properties of the system. Ado fluxes, obtained from coefficients of the first-order term in least-squares parabolas fitted to the uptake data were plotted against Ado concentration (Fig. 16).

[12] Mean $\pm$ S.E. from 4 experiments

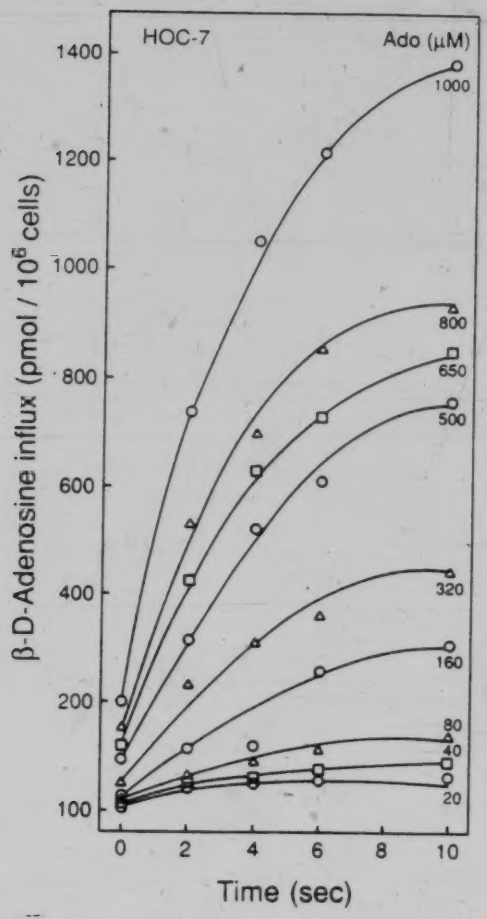


Fig. 15. Time courses of Ado uptake in HOC-7 cells. Intervals of Ado uptake in replicate monolayers were started by addition of transport medium containing ^{3}H-Ado, and ended by immersing the cultures in ice-cold PBS medium. Cell content of ^{3}H-Ado was quantitated as described in Fig. 8. Final permeant concentrations are indicated. The data shown are means of triplicate assays and are typical of 3 similar experiments.

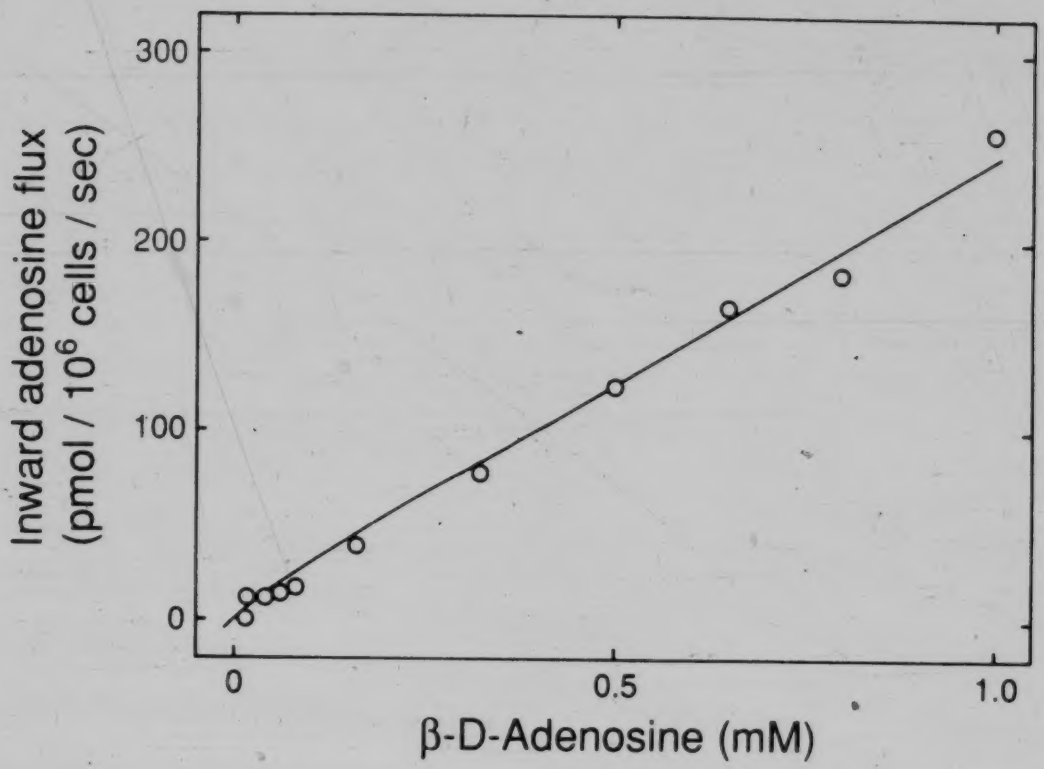


Fig. 16. Concentration dependence of Ado fluxes in HOC-7 cells. ^{3}H-Ado fluxes were determined as initial rates from time courses of uptake of graded Ado concentrations (Fig. 15) by the parabola-fitting method described in Materials and Methods. The data shown are means of triplicate assays and are typical of 3 similar experiments.

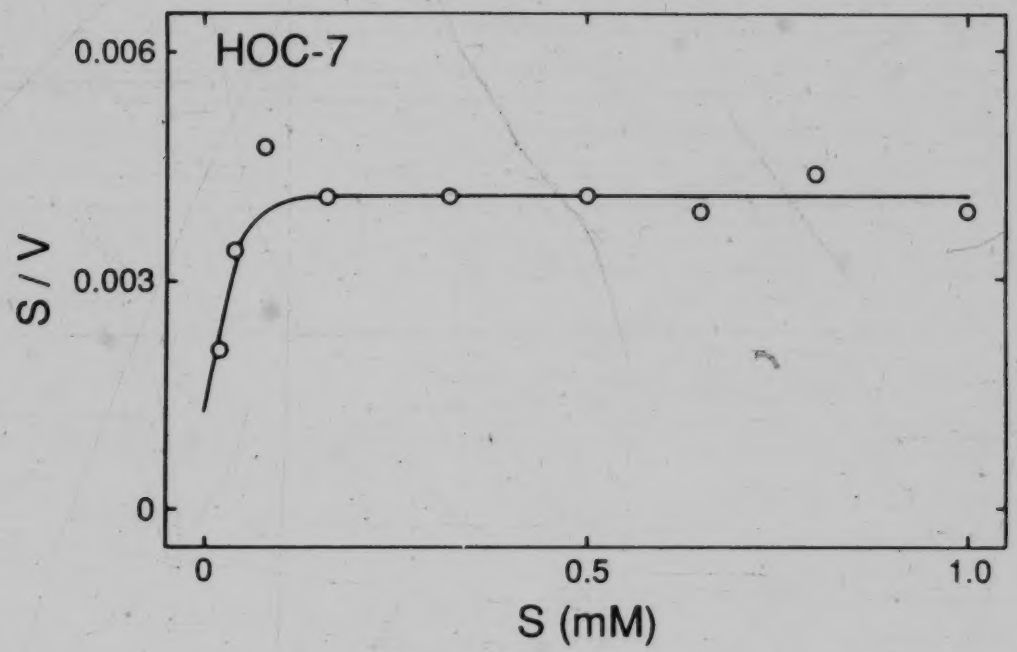


Fig. 17. Flux-concentration relationships in permeation of Ado in HOC-7 monolayer cells. Flux-concentration data from Fig. 16 were analysed by the Hanes-Woolf method (Hanes, 1932; Woolf, 1932). The biphasic nature of the plot indicates that two processes contribute to Ado influx, one being saturable and transporter-mediated and the other transporter-independent. These data are means of triplicate assays and are typical of 3 similar experiments. Extrapolation of the saturable component yielded mean ($\pm$ S.E.) K_m and V_{max} values of 28 $\pm$ 9 μM and 27.5 $\pm$ 7.8 pmoles/10^6 cells/sec representing the 3 similar experiments.

The parabola fitting procedure is equivalent to the application of theoretical tangents to the uptake curves at zero-time (Harley *et al.*, 1982; Belt, 1983). The rate-concentration plot was initially curved, but did not saturate, even at high permeant concentrations. Fig. 17 is a Hanes-Woolf plot of the velocity-concentration data of Fig. 16. The Hanes-Woolf analysis suggests that two permeation processes contribute to Ado influx in these cells; the initial linear portion of the Fig. 17 plot indicates the participation of a transporter-mediated process, whereas the later horizontal component of the Fig. 17 plot implies the participation of a non-mediated process (Neame and Richards, 1972).

Because transporter-independent fluxes of physiological nucleosides are low in various animal cell types (Paterson *et al.*, 1987; Plagemann *et al.*, 1988), the non-saturable component of Ado fluxes found in HOC-7 cells was much larger than might be expected. The saturable component of Ado permeation yielded the apparent kinetic constants: K_m, 28 ± 9 μM and V_{max}, 27.5 ± 7.8 pmoles/10^6 cells/sec. That K_m value is in general agreement with those obtained by quenched-flow or "inhibitor-stop" methods for Ado fluxes in several types of cultured neoplastic cells in this laboratory (Paterson *et al.*, 1984,1985; Paterson and Cass, 1986), and with values reported by Chello *et al.*, (1983). In contrast, K_m values reported by Plagemann and co-workers are 2- to 5-fold higher (Lum *et al.*, 1979; Plagemann and Wohlhueter, 1980; Plagemann *et al.*, 1988). These differences may have a methodological basis.

Kinetics of NBMPR-Insensitive Adenosine Transport

To characterise the NBMPR-insensitive component of Ado permeation observed in HOC-7 cells, Ado fluxes were evaluated in the presence of 10 μM NBMPR, a concentration at which the NBMPR-sensitive facilitated diffusion system is blocked. The experimental procedure was identical to that used in the experiments of Figs. 16 to 18. Replicate monolayers were incubated at 22° in transport medium containing 10 μM NBMPR for 15 min

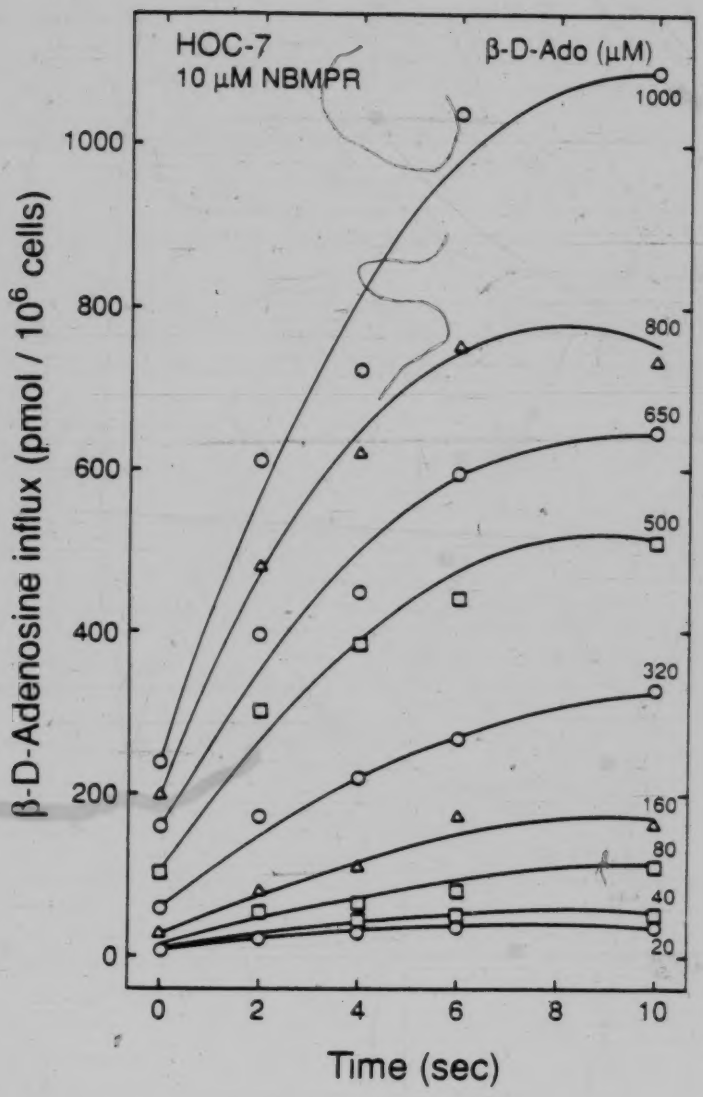


Fig. 18. Time courses of Ado uptake by HOC-7 cells in the presence of NBMPR. Replicate HOC-7 monolayers were incubated at 22° with 10 μM NBMPR in transport medium for 15 min before assay of Ado fluxes as in Fig. 8. Intervals of Ado uptake were started by addition of transport medium containing graded concentrations of ^{3}H-Ado and 10 μM NBMPR to culture dishes and were terminated by immersion of dishes in ice-cold PBS. The data shown are means of triplicate assays and are typical of 3 similar experiments.

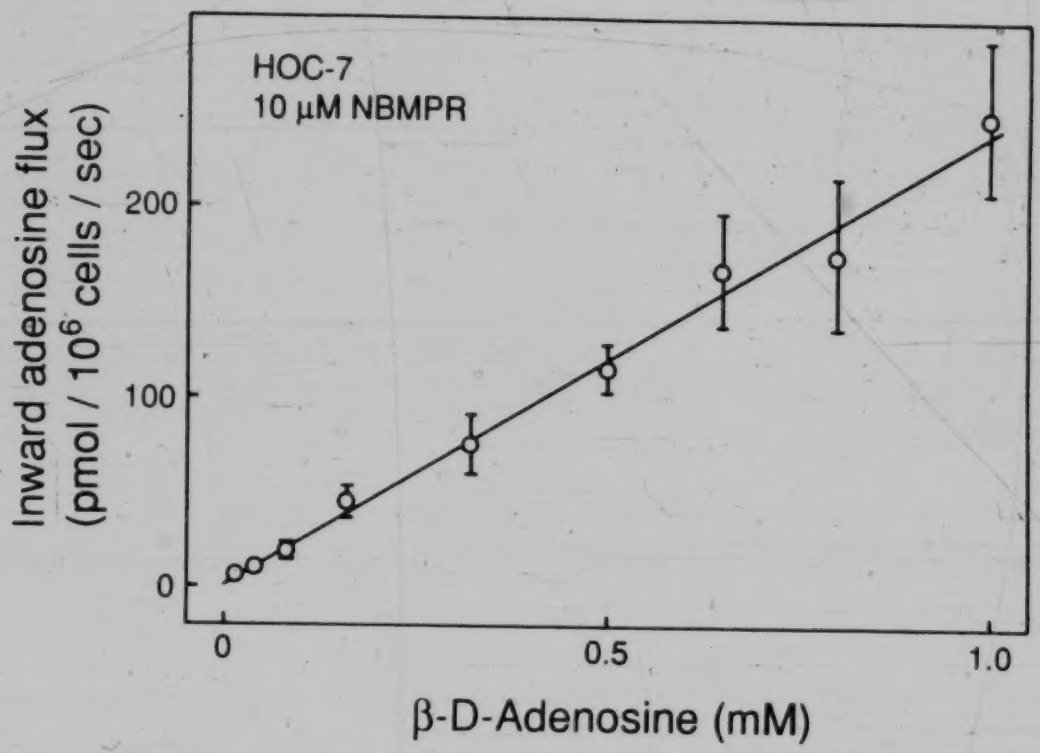


Fig. 19. Concentration dependence of Ado fluxes in HOC-7 cells in the presence of NBMPR. Ado fluxes in replicate HOC-7 monolayers, were determined from time course data of ^{3}H-Ado uptake by the parabola-fitting method, as described in Fig. 8 and Materials and Methods. The data shown are means ± S.E. from 3 similar experiments in which assays were conducted in triplicate.

prior to assays of inward Ado fluxes at graded Ado concentrations in the presence of 10 μM NBMPR (final concentration). Fig. 18 presents time courses of Ado uptake by HOC-7 monolayers under those conditions and initial rates from the time courses are plotted against substrate concentration in Fig. 19. The fluxes did not saturate, but increased proportionately with susbstrate concentration. This result indicates that the NBMPR-insensitive component of Ado fluxes in HOC-7 cells is a transporter-independent process.

Inhibition of Inward Ado Fluxes by Competing Substrates

One of the important criteria for recognizing the participation of a transport system in the entry into cells of a given permeant is flux inhibition by related permeants (Wilbrandt, 1975; Widdas, 1988). Conversely, the absence of flux inhibition by related permeants argues for participation of a transporter-independent permeation process.

The facilitated diffusion NT system accepts both physiological nucleosides and a variety of nucleoside analogues as substrates (Paterson and Cass, 1986). Several workers have used the inhibition of nucleoside fluxes by various competing nucleosides to demonstrate the participation of NBMPR-insensitive carrier systems in those fluxes (Belt, 1983; Jarvis and Young, 1986).

In the experiments summarised in Fig. 20, transport medium contained ^{3}H-Ado (10 μM) and graded concentrations of the potentially competitive nucleosides, Ado, Urd, dAdo, thymidine (dThd), inosine (Ino) or tubercidin (Tu). Inward fluxes of ^{3}H-Ado in HOC-7 monolayer cells were assayed as described in Fig. 8 and in Materials and Methods. Ado fluxes determined from time course data in the presence of competing nucleosides were expressed as percentages of control fluxes, measured in the absence of competing nucleosides. The individual presence of these purine and pyrimidine nucleosides in the Ado flux assays in concentrations of up to 10 mM inhibited Ado fluxes by no more than 30%, suggesting that transporter-mediated fluxes are a minor component of Ado permeation in HOC-7 cells.

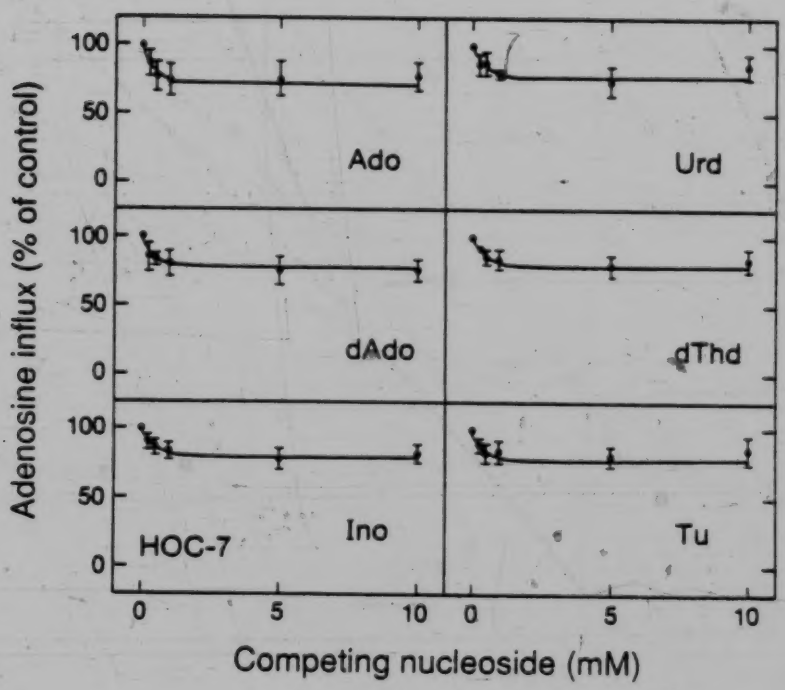


Fig. 20. Influence of competing nucleosides on inward fluxes of Ado in HOC-7 cells. Intervals of uptake were started by addition to replicate HOC-7 monolayer cultures of transport medium containing 10 μM ^{3}H-Ado and graded concentrations of non-isotopic nucleosides; intervals were ended by immersion of culture dishes in ice-cold PBS. Ado fluxes were determined from time course data by curve-fitting as in Fig. 7. Fluxes in the presence of graded concentrations of competing non-isotopic nucleosides were expressed as percentages of Ado fluxes measured in the absence of competing permeants (4.6 ± 0.7 pmol/10^6 cells/sec, 100%). The data shown are means ± S.E. from 3 similar experiments in each of which assays were performed in triplicate.

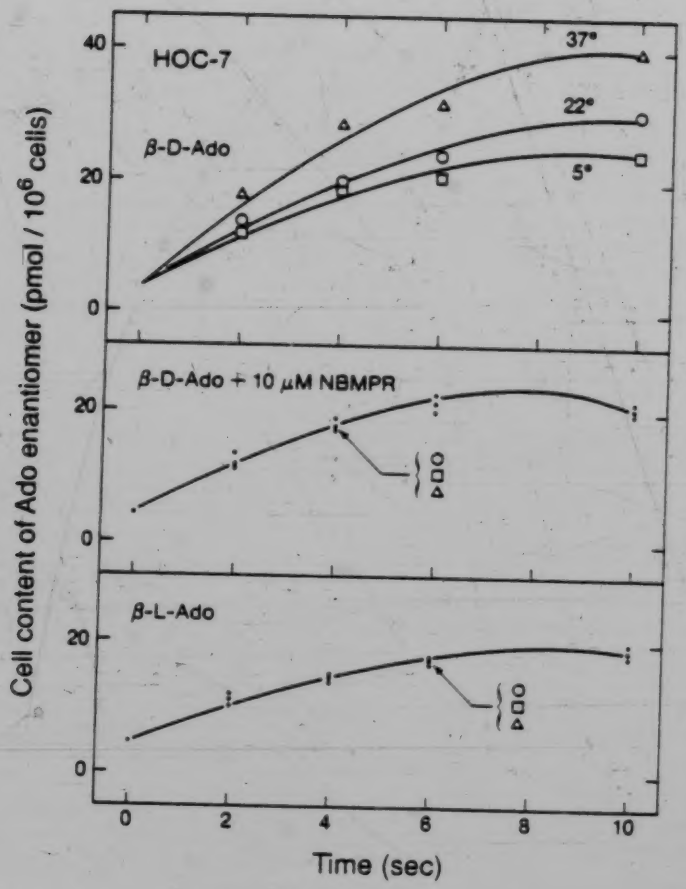


Fig. 21. Temperature dependence of Ado influx in HOC-7 cells. Replicate HOC-7 monolayer cultures were incubated at the specified temperatures for 15 min prior to assays of nucleoside uptake. Intervals of influx were started by addition of transport medium containing 15 μM ^{3}H-Ado at the specified temperatures, and ended by immersing monolayers into ice-cold PBS. The data shown are means of triplicate assays and are typical of 3 similar experiments.

Temperature-dependence of Ado Transport

Facilitated diffusion fluxes exhibit greater temperature dependence than simple diffusion fluxes (Berlin and Oliver, 1975; Goldenberg and Begleiter, 1984). This difference in temperature dependence has been used by Zimmerman and co-workers (1987), as a criterion for distinguishing between simple diffusion and facilitated diffusion processes in the permeation of AZT and thymidine in human erythrocytes. However, Plagemann *et al.*, (1988) have pointed out that the non-mediated permeation of nucleosides and nucleobases in some cell types is highly temperature-dependent, with Q_{10} values that may not be distinguishable from those of facilitated diffusion transporters. In the experiments depicted in the upper panel of Fig. 21, Ado fluxes in HOC-7 cells were measured at 5°, 22° and 37°C. Ado fluxes increased from 4.5 ± 0.4 pmol/10^6 cells/sec at 5°, to 6.8 ± 0.1 pmol/ 10^6 cells/sec at 37° (means ± S.E. from 3 experiments). In contrast, the experiments illustrated in the middle panel of Fig. 21 show that, when measured in the presence of 10 μM NBMPR (thus blocking Ado permeation by the facilitated diffusion mechanism), Ado fluxes were virtually independent of temperature (4.0 ± 0.1 pmol/10^6 cells/ sec at 5°, 4.6 ± 0.3 pmol/ 10^6 cells/sec at 37°), suggesting that those are transporter-independent fluxes.

Influx of β-L-Adenosine

Transporter polypeptides in animal cell membranes discriminate between "mirror-image" isomers of permeant molecules (Hofer, 1977; Finean, 1984). Enantiomeric selectivity has been reported for transport of D-glucose in mammalian cells, and L-glucose fluxes have been used in some cell types as a measure of transporter-independent glucose permeation. Work in this laboratory has indicated that β-L-Ado, the enantiomer of the naturally occuring nucleoside, β-D-Ado, is poorly transported by the facilitated diffusion

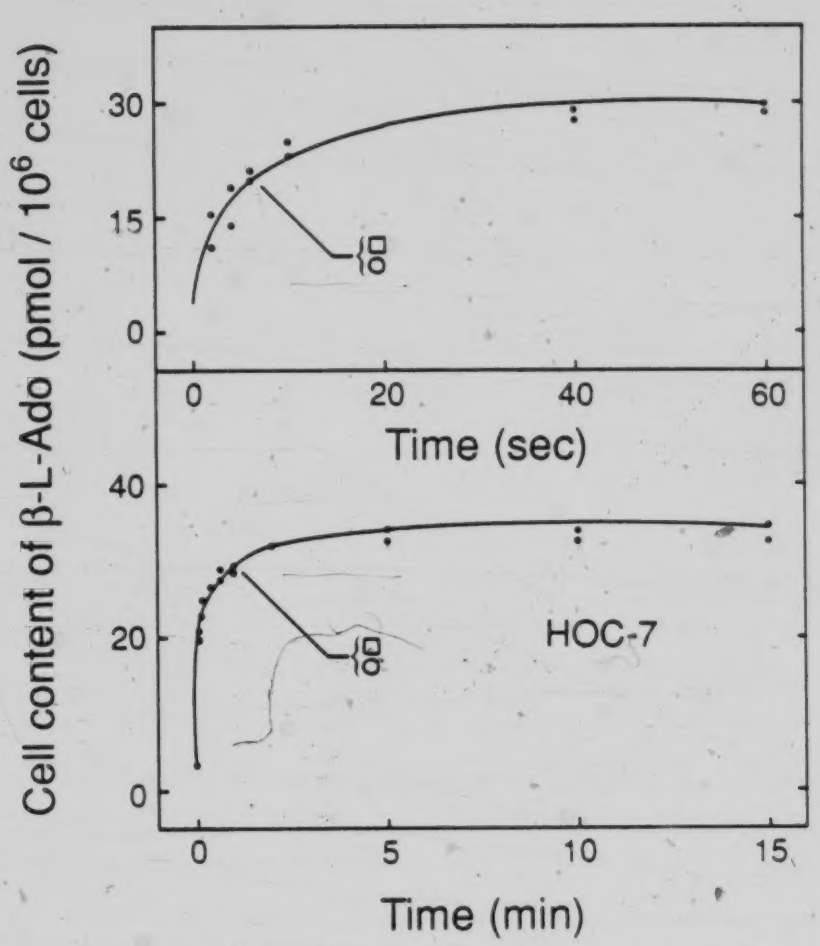


Fig. 22. Time courses for the uptake of β-L-Ado in HOC-7 cells. Intervals of β-L-Ado uptake in replicate monolayers were started by addition to cultures of transport medium containing 15 μM ^{3}H-β-L-Ado with (□) or without (■) 10 μM NBMPR. Uptake intervals were ended by immersing cultures in ice-cold PBS medium. Cells were incubated at 22° for 15 min with or without 10 μM NBMPR prior to assays of β-L-Ado uptake. The data shown are means of triplicate assays; similar results were obtained when the experiments were repeated.

systems of mouse erythrocytes [13] , L1210 mouse leukaemia cells (Dagnino, 1988), rat hepatocytes (Awumey, 1989) and SK-OV-3 ovarian carcinoma cells (this study). β-L-Ado is also a poor permeant for the concentrative, sodium-linked NT systems of IEC-6 intestinal epithelial cells[14] , L1210 cells (Dagnino, 1988) and rat hepatocytes (Awumey, 1989).

Fig. 22 illustrates experiments in which fluxes of 15 μM ^{3}H-β-L-Ado were measured in HOC-7 monolayer cultures in the presence and absence of 10 μM NBMPR. Influx was rapid at early time points and was not inhibited by NBMPR. Equilibrium between intracellular and extracellular ^{3}H-β-L-Ado, which which had nearly been attained at 1 min (Fig. 22, lower panel), was complete within 5 min. β-L-Ado influx was non-concentrative since intracellular and extracellular concentrations were approximately equal at equilibrium.[15] To investigate the possibility that the apparent permeation of ^{3}H-β-L-Ado might represent the association of β-L-Ado molecules with Ado receptors in the cell membrane and not transmembrane transport, or that the apparent permeation process might be transporter-mediated, inward fluxes of β-L-Ado were measured as described in Fig. 20, in the presence of graded concentrations of the competing permeants, β-D-Ado and β-D-dAdo. Results illustrated in Fig. 23 show that these permeants failed to inhibit β-L-Ado fluxes in concentrations of up to thousand-fold molar excesses, and suggest the participation of a transporter-independent permeation process in the entry of β-L-Ado into HOC-7 cells.

Kinetics of β-L-Adenosine Influx

Fluxes of various permeant molecules across plasma membranes of some animal cell types achieve a limiting value as permeant concentration progressively increases. Such rate saturation is convincing demonstration of a transporter-mediated process (Wilbrandt, 1975; Stein, 1986). Time course data for the permeation of graded concentrations of ^{3}H-β-L-Ado

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[13] W. P. Gati *et al.*, submitted for publication

[14] Jakobs, E. S., and Paterson, A. R. P., unpublished data

[15] The intracellular water space of trypsin-detached cells was 2.8 ± 0.5 μl/10^{6} cells (Mean ± S.E. from 10 experiments).

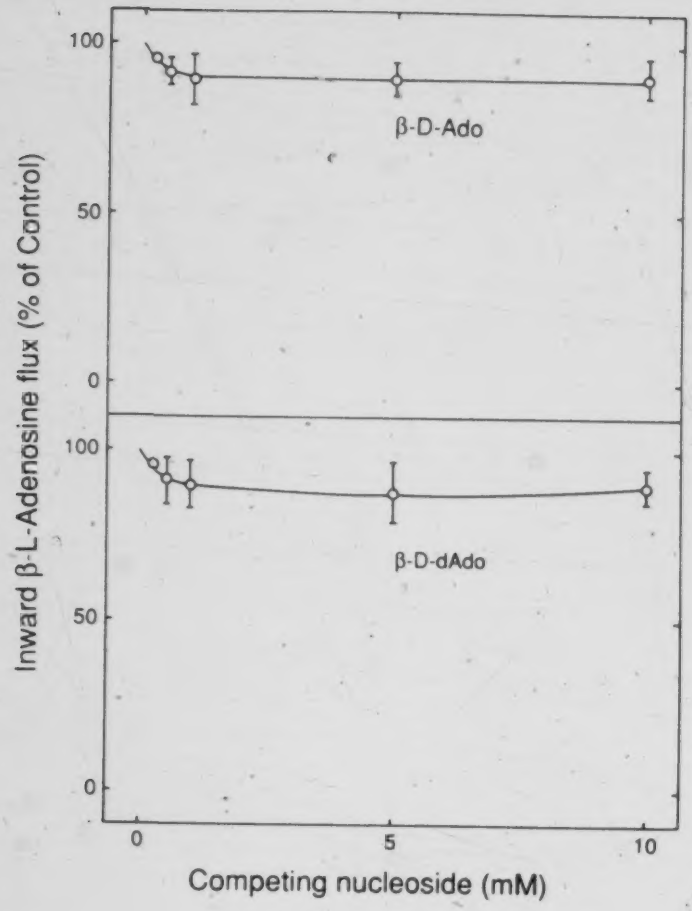

Fig. 23. Failure of β-D-Ado and β-D-dAdo to inhibit fluxes of ^{3}H-β-L-Ado in HOC-7 cells. β-L-Ado fluxes were measured as initial rates of time courses of 10 μM ^{3}H-β-L-Ado influx in replicate HOC-7 monolayers by the procedure outlined in Fig. 7. Fluxes measured in the presence of graded concentrations of non-isotopic β-D-Ado or β-D-dAdo are expressed as percentages of a control flux measured in the absence of the latter, "competimg" nucleosides. The control flux (100%) in this study was 3.1 ± 0.3 pm/10^{6} cells/sec. These data are means ± S.E. from 3 similar experiments in each of which assays were performed in triplicate.

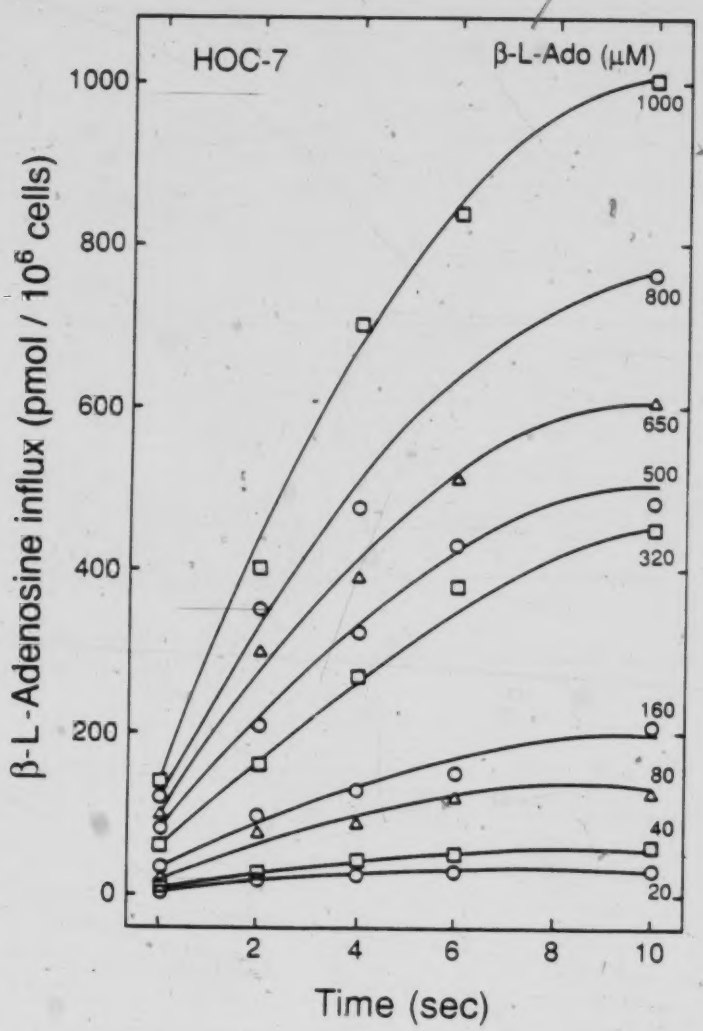

Fig. 24. Time courses of β-L-Ado uptake in HOC-7 cells. Assays of ^{3}H-β-L-Ado uptake in replicate monolayer cultures were performed as in Fig. 15. The data shown are means of triplicate assays and are typical of 3 similar experiments.

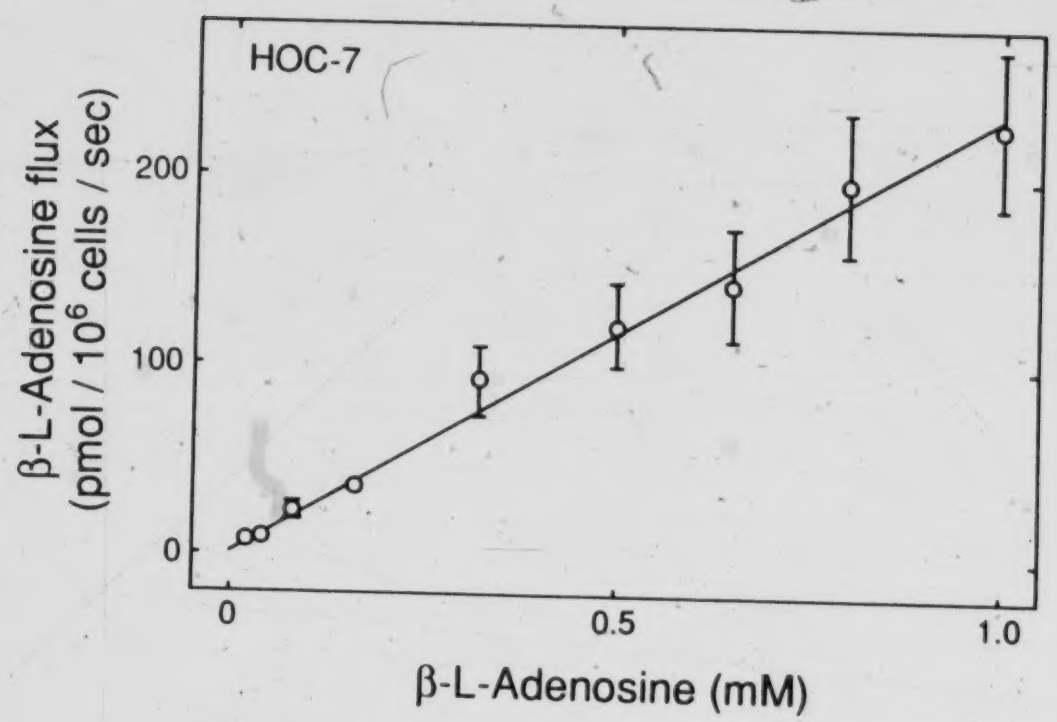

Fig. 25. Concentration dependence of inward fluxes of β-L-Ado in HOC-7 cells. β-L-Ado fluxes in replicate HOC-7 monolayers were determined as initial rates from time course data of ^{3}H-β-L-Ado as described in Fig. 7 and in Materials and Methods. The data shown are means ± S.E. from 3 similar experiments in which assays were conducted in triplicate.

in HOC-7 monolayers are shown in Fig. 24 and the concentration dependence of β-L-Ado fluxes derived from these data is depicted in Fig. 25. Such fluxes are seen to be directly proportional to β-L-Ado concentration, indicating a transporter-independent process.

Metabolism of Nucleosides

Nucleated cells utilise Ado molecules for nucleotide synthesis. As well, Ado molecules are catabolised to inosine and purine bases (Fox and Kelley, 1978) in those cells. β-L-Ado is a poor substrate for enzymes of Ado metabolism in animal cells (Asai *et al.*, 1967). Studies of β-L-Ado metabolism were a valuable tool in authenticating the chemical nature of isotopic β-L-Ado stocks utilised in this study. A non-metabolised nucleoside permeant in HOC-7 cells was needed to load cells for countertransport experiments described in a subsequent section.

The metabolism of β-D-Ado, β-L-Ado and formycin B was studied in HOC-7 cells by (i) incubation of monolayers with 100 μM isotopic nucleoside for appropriate time intervals, and (ii) chromatographic analysis of TCA extracts of the cells. After neutralization, TCA extracts were chromatographed on thin layers of PEI cellulose using distilled water as the solvent. Chromatography on PEI cellulose-borate sheets (Drack and Novack, 1973) was used for the analysis of β-D-Ado metabolites. Histograms describing the distribution on chromatograms of ^{3}H-labelled nucleosides and metabolites thereof in cell extracts are shown in Fig. 26. These data show the absence of β-L-Ado and formycin B metabolites in HOC-7 cells after 60 min of incubation at 22°C. Under these conditions, β-D-Ado was extensively phosphorylated, without apparent conversion to inosine during 30 sec of incubation.

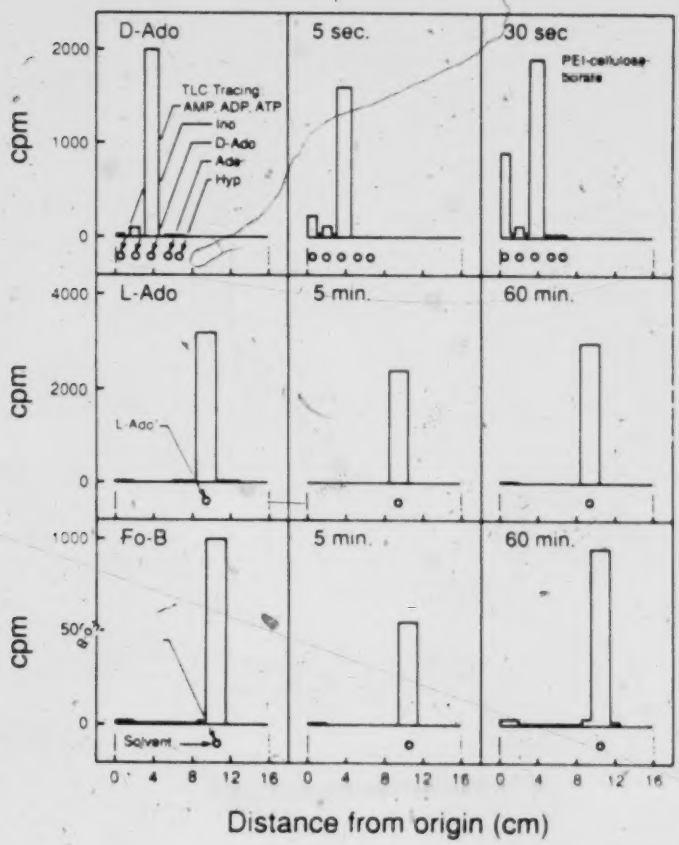

Fig. 26. Metabolism of purine nucleosides in HOC-7 cells. The histograms depict the chromatographic distribution of ^{3}H-activity in TCA extracts of HOC-7 cells following incubation with ^{3}H-nucleosides. Monolayers were extracted with 1.0 ml cold 0.4 M TCA and after neutralization, extracts were chromatographed on PEI cellulose TLC sheets, or on PEI cellulose-borate in the instance of β-D-Ado, together with 10 nmol portions of the carrier substances indicated. Sections of chromatogram lanes (0.5 cm) were assayed for ^{3}H-activity. Tracings of a typical set of chromatograms viewed under u/v light are shown. Broken lines indicate solvent fronts.

Countertransport

A rigorous criterion for recognition of a facilitated diffusion permeation process is the demonstration of countertransport or counterflow (Rosenberg and Wilbrandt, 1957; Stein, 1986; Widdas, 1954).

In the present study, outward countertransport of formycin B driven by β-D-Ado influx, but not by influx of β-L-Ado, was shown in the experiment of Fig. 27. Replicate HOC-7 monolayer cells were first "loaded" with formycin B by incubation (22°, 45 min) with 1.4 ml of 10 μM ^{3}H-formycin B in transport medium. At equilibrium, the cell content of ^{3}H-formycin B was 32.4 ± 2.7 pmol/10^{6} cells (mean ± S.E. in 3 experiments). At time-zero, 0.6 ml portions of 4 mM solutions of the non-isotopic test nucleosides in transport medium were added to the suspensions with rapid mixing (final concentrations of test nucleosides, including formycin B in the external medium were 1.2 mM); after designated time intervals, monolayers were assayed for their content of ^{3}H-formycin B. The formycin B content of monolayer cells declined with time when medium contained 7 μM ^{3}H-formycin B without additives (control, 0.6 mM transport medium added) or 1.2 mM non-isotopic nucleosides. When the medium contained β-L-Ado, the time course of decline in cellular formycin B (Fig. 27) was not significantly different ($p > 0.05$) from that of control cultures. The decline in cellular formycin B content was more rapid when medium contained formycin B or β-D-Ado. The decline in cellular ^{3}H-formycin B was more rapid when external medium contained non-isotopic β-D-Ado than when it contained formycin B, perhaps because β-D-Ado is rapidly phosphorylated in HOC-7 cells (Fig. 26), leading to intracellular trapping and stimulating more rapid and sustained efflux of ^{3}H-formycin B. This experiment demonstrated clearly that influx of both formycin B and β-D-Ado is transporter-mediated, and that the influx of β-L-Ado is transporter-independent.

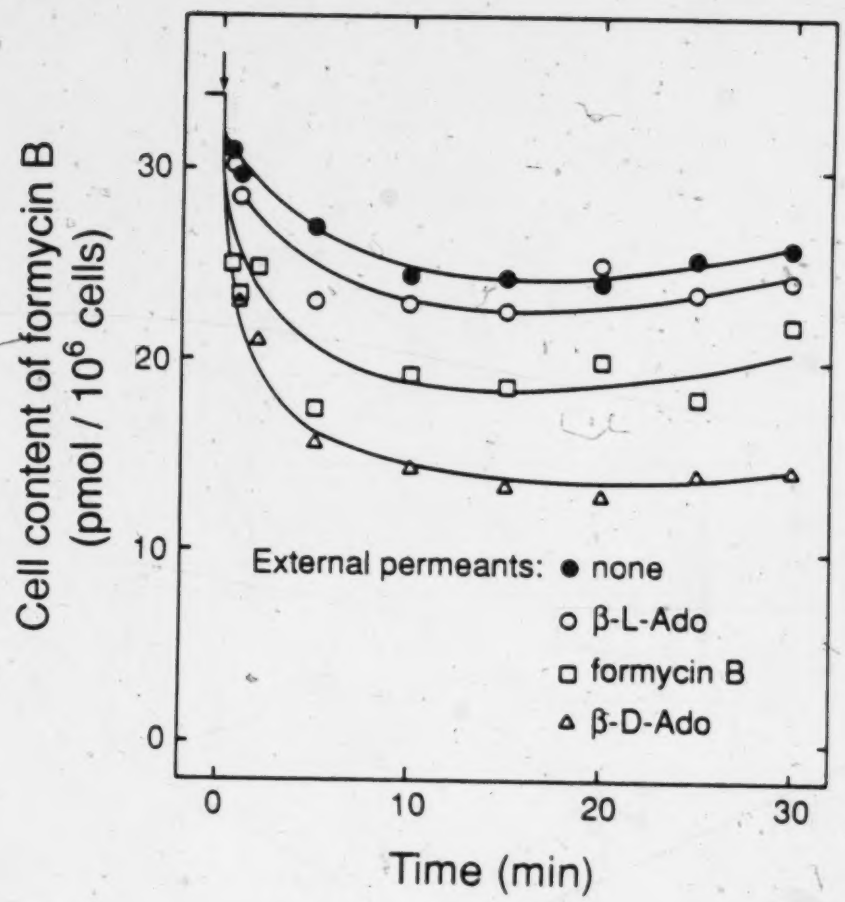

Fig. 27. Countertransport of formycin B and D-Ado in HOC-7 cells. Replicate monolayers were incubated (22°, 45 min) with 1.4 ml transport medium containing 10 μM ^{3}H-formycin B and, at time-zero (arrow), 0.6 ml portions of transport medium containing 4 mM non-isotopic β-L-Ado (○), formycin B (□), β-D-Ado (△) and no nucleosides (●) were added with mixing. After the intervals indicated at 22°, monolayers were assayed as in Fig. 8 for cell content of ^{3}H-formycin B. At time-zero (indicated by the vertical arrow), cell content of ^{3}H-formycin B was 32.4 ± 2.7 pmol/10^6 cells/sec (mean ± S.E. from 3 experiments). The data shown are the means of triplicate assays and are typical of 3 similar experiments.

Transporter-independent Influx of Nucleosides

While facilitated diffusion nucleoside transporters accept as permeants the physiologic nucleosides and a diverse array of nucleoside analogues (Cass *et al.*, 1987; Paterson and Cass, 1986; Paterson *et al.*, 1987), some of the recently described sodium-linked, concentrative NT systems have narrower substrate specificities. Thus, the sodium-linked NT system of L1210 mouse leukaemia cells does not accept thymidine (Dagnino, 1988), and two sodium-linked NT systems distinct in their substrate specificities have been described in mouse intestinal epithelial cells (Vijayalakshmi and Belt, 1988). As well, simple diffusion of Ado has been described in nucleoside-impermeable sheep erythrocytes (Young, 1978). The present study has shown that in HOC-7 cells, a large fraction (approximately 70%) of inward Ado fluxes is not transporter-mediated. The substrate specificity of the apparently transporter-independent permeation route in HOC-7 cells was investigated in rate-concentration studies of the fluxes of several physiological nucleosides to determine if rate saturability was detectable. Inward fluxes of ^{3}H-labelled Ino, Urd, dThd, dAdo and Guo (guanosine) were assayed in NBMPR-containing transport medium, as described in earlier sections. Fig. 28 illustrates the flux-concentration relationships obtained with HOC-7 monolayer cells under these conditions; these data provide no evidence of flux saturability at permeant concentrations up to 1 mM. These results indicate that in HOC-7 cells , transporter-independent processes contribute substantially to the entry of Ino, Urd, dThd, dAdo and Guo.

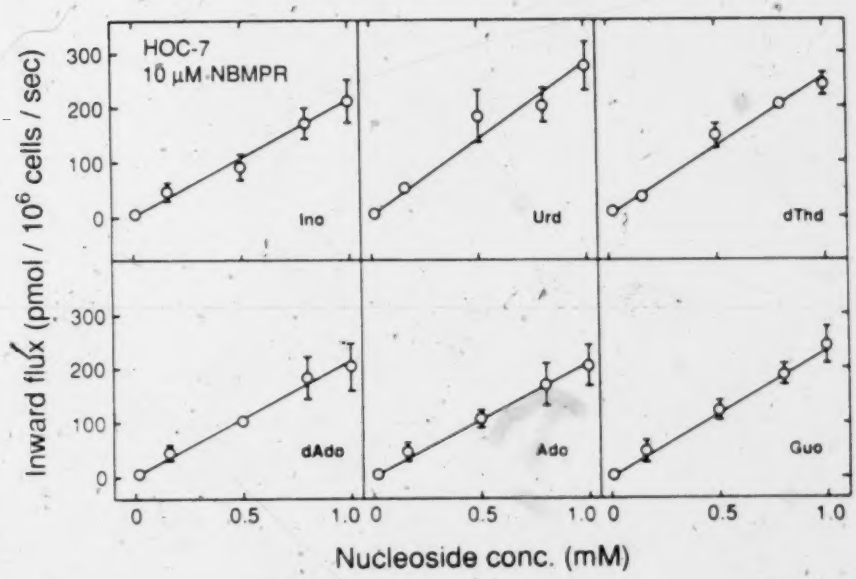

Fig. 28. Concentration dependence of inward nucleoside fluxes in HOC-7 cells in the presence of NBMPR. Time courses of ^{3}H-nucleoside uptake in replicate HOC-7 cultures were determined as described in Fig. 18. Nucleoside fluxes were determined from such time course data and plotted against substrate concentration. Values shown are means ± S.E. from 3 experiments, each performed with triplicate assays.

Octanol-PBS Partition Coefficients

Partition coefficients in *n*-1-octanol-PBS systems are thought to reflect the solubility of test substances in membrane lipids (Leo *et al.*, 1971; Bech-Hansen *et al.*, 1972). The octanol-PBS partition coefficients of nucleosides summarised in Fig. 29 were determined by mixing octanol with an equal volume of PBS containing ^{3}H-labelled nucleosides (Ado, Urd, dThd, Ino, dAdo or Guo). Mixtures were kept in motion at 22° overnight on a gyratory shaker and samples from the two phases were assayed for ^{3}H-activity, yielding the results of Table 9. Substitution of water for PBS yielded similar data. That table also compares the partition coefficents of the nucleosides tested with their permeation rate constants[16] in HOC-7 cells. As is evident in Fig. 29, nucleoside lipophilicity (partition coefficient) did not correlate with rates of nucleoside permeation.

Effects of Non-specific Transport Inhibitors

The thiol reagents, *p*-CMBS and NEM, which, respectively, react with nucleoside transport-associated sulfhydryl groups in the extracellular and cytoplasmic domains of the cell membrane, have been shown to inhibit NBMPR-insensitive nucleoside transport (Belt and Noel, 1985; Jarvis and Young, 1986), and the sodium-linked nucleoside transport systems (Dagnino, 1988). Phlorizin and its aglycone, phloretin, inhibit the malarial parasite-induced permeability pathways in erythrocytes (Ginsburg and Stein, 1987b).

[16] Slopes of the flux-concentration relationships depicted in Fig. 28. These derivations are a measure of the transporter-independent permeation rates of nucleosides in HOC-7 cells, and approximate permeability coefficients of such nucleosides. Determination of permeability coefficients requires knowledge of the surface area of the cells under investigation (Plagemann *et al.*, 1988).

Table 9. Partition coefficients and rate constants for transporter-independent fluxes of physiological nucleosides in HOC-7 cells.

Octanol-PBS partition coefficients were determined as described in Materials and Methods. Slopes of the Fig. 28 flux-concentration plots are expressed as permeation rate constants of the test nucleosides.

Nucleoside	Molecular weight	Octanol-PBS partition coefficient	Permeation rate constant (pmol/s/μM/10⁶cells)
D-Adenosine	267	0.089 ± 0.002[1]	0.212 ± 0.019[2]
2'-Deoxyadenosine	251	0.196 ± 0.007	0.216 ± 0.022
Guanosine	283	0.020 ± 0.002	0.232 ± 0.079
Inosine	268	0.015 ± 0.001	0.216 ± 0.092
Uridine	244	0.013 ± 0.003	0.280 ± 0.050
Thymidine	242	0.071 ± 0.001	0.256 ± 0.047

[1] Values are means ± S.D. of 6 determinations

[2] Means ± S.E. from the 3 experiments referred to in Fig. 28.

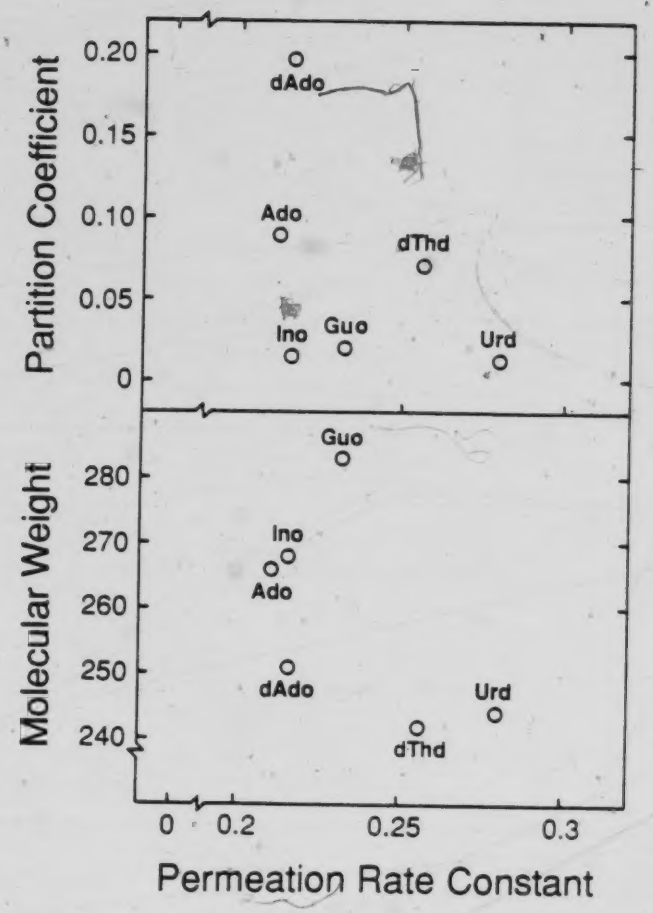

Fig. 29. Lack of correlation of molecular weights and partition coefficients of physiological nucleosides with their rate constants for transporter-independent permeation in HOC-7 cells. Partition coefficients of the ^{3}H-labelled test nucleosides, Ado, dAdo, Ino, Guo, dThd and Urd, as well as their permeation rate constants in HOC-7 cells, were determined as described in Materials and Methods and in Table 9.

Also, furosemide, an inhibitor of sodium and chloride co-transport systems in animal cells, has been shown to inhibit an apparently volume-sensitive route of nucleoside permeation in eel erythrocytes (J. D. Young, unpublished).

The above-mentioned compounds were evaluated as potential inhibitors of the transporter-independent influx pathway for nucleosides in HOC-7 cells, using β-L-Ado fluxes to measure nucleoside permeation by that route. Table 10 shows that such fluxes were not inhibited by high concentrations of these agents (500-1000 μM).

Transporter-independent Adenosine Influx in Ovarian Carcinoma Cells

The possible expression of the transporter-independent, inhibitor-insensitive nucleoside influx pathway in lines of human ovarian carcinoma cells other than HOC-7 was explored in experiments summarised in Fig. 30. The HOC-1 cell line was initiated from a sample of ascitic fluid from a patient, eight months prior to the establishment of the HOC-7 line in the same manner from the same patient (Buick *et al.*, 1985). The HEY line was initiated from a disaggregated, human papillary cystadenocarcinoma of the ovary by passage in immune-deficient mice (Selby *et al.*, 1980) prior to passage in culture. β-L-Ado fluxes in monolayer cultures of these HOC-1, HOC-7 and HEY cells, assayed in the experiments of Fig. 30, were similar to those of β-D-Ado. β-L-Ado fluxes were resistant to NBMPR inhibition, and are seen to be a measure of transporter-independent permeation. Fig. 30 shows that time courses of β-L-Ado uptake in HOC-1, HOC-7 and HEY cells were similar. There was very little entry of β-L-Ado into SK-OV-3 cells which expressed virtually no transporter-independent component of Ado permeation. ^{14}C-Sucrose was almost completely excluded from these cells, indicating that the transporter-independent nucleoside fluxes are not attributable to non-specific leakage of permeant across the plasma membrane.

Table 10. Failure of several transport inhibitors to impede inward fluxes of β-L-Ado in HOC-7 cells. Inward fluxes of ^{3}H-β-L-Ado (15 μM) were determined in the absence and presence of several putative inhibitors. HOC-7 monolayers were incubated for 10 min with inhibitors prior to assay of inward fluxes of β-L-Ado from transport medium containing the inhibitors at the designated concentrations. Such fluxes in the presence of inhibitors were expressed as percentages of control (3.5 ± 0.5 pm/10^{6} cells/sec, 100%) fluxes.

Inhibitor	Concentration (mM)	β-L-Ado flux (% of control)[2]
pCMBS[1]	0.5	100 ± 2
NEM[1]	0.5	98 ± 5
Phlorizin	0.5	97 ± 4
Phloretin	0.5	94 ± 7
Furosemide	1.0	92 ± 7
SITS[1]	0.5	95 ± 3

[1] Abbreviations are defined on page xiv.

[2] Mean ± S.E. from 4 experiments.

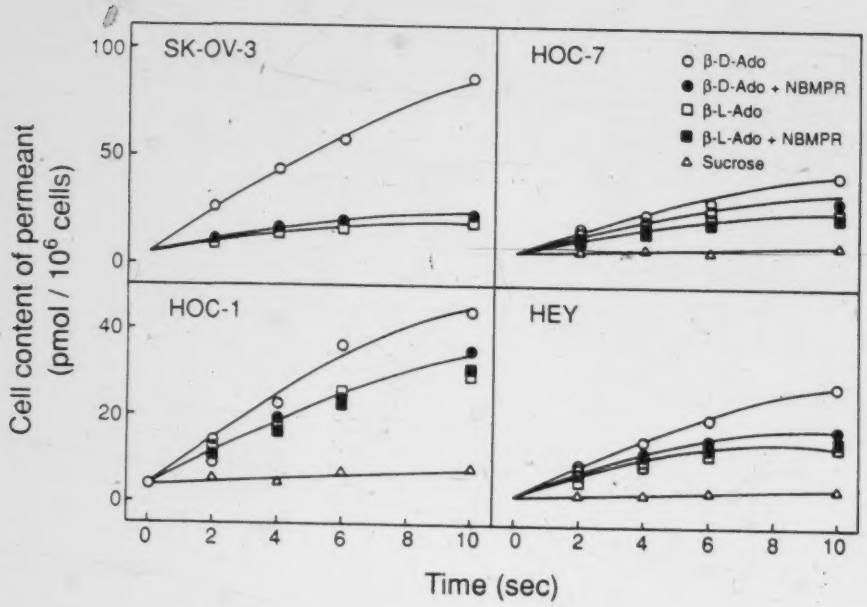

Fig. 30. Ado and sucrose permeation in cultured human ovarian carcinoma cells. Replicate cultures were incubated for 15 min with or without graded NBMPR concentrations prior to assays of permeant uptake. Intervals of uptake were started by flooding replicate monolayers with transport medium containing ^{3}H-labelled β-D-Ado, β-L-Ado, or sucrose with or without 10 μM NBMPR as indicated. Intervals of permeant uptake were ended by immersion of culture dishes in ice-cold PBS. Cell content of permeant was quantitated as described in Fig. 8. The data shown are means of triplicate assays and are typical of results from 3 similar experiments.

G. HEY Cells

Cells of the HEY line, which grow well in culture (doubling time 17 hrs) were investigated further to explore nucleoside permeation characteristics. The low sensitivity of Ado fluxes in HEY cells to NBMPR apparent in Fig. 31 suggested permeation of Ado by a transporter-independent process. Ado flux-concentration relationships explored in the presence of 10 μM NBMPR in the experiment of Fig. 32 indicated a linear dependence of transporter-independent Ado fluxes on Ado concentration. Together with the findings illustrated in Fig. 30, these results are an indication of the similarities in the nucleoside permeation processes of HEY and HOC-7 cells. Transporter-independent components of nucleoside fluxes may well be expressed in other lines of human ovarian carcinoma cells.

H. Growth Inhibition Studies

Earlier reports from this laboratory demonstrated that NBMPR at concentrations of 5-8 μM protected particular cell lines (RPMI 6410 human lymphoblastoid cells (Paterson *et al.*, 1979b) and S49 mouse lymphoma cells (Cass *et al.*, 1981)) against lethal concentrations of tubercidin and other cytotoxic nucleosides during several days of culture. The experiments summarised in Fig. 33 measured concentrations of FaraA and araC that reduced proliferation rates of SK-OV-3, HOC-7 and HEY cultures by 50% (IC_{50}) in the presence and absence of 10 μM NBMPR. Culture media contained graded concentrations of the toxicants with or without 10 μM NBMPR, and cell numbers were enumerated after 72 hours of culture. In SK-OV-3 cultures, NBMPR increased the IC_{50} of araC from 7 μM to 15 μM, but had no effect on the IC_{50} of FaraA. IC_{50} values of araC and FaraA were not affected by NBMPR in HOC-7 and HEY cells, both of which express a major transporter-independent nucleoside permeation pathway.

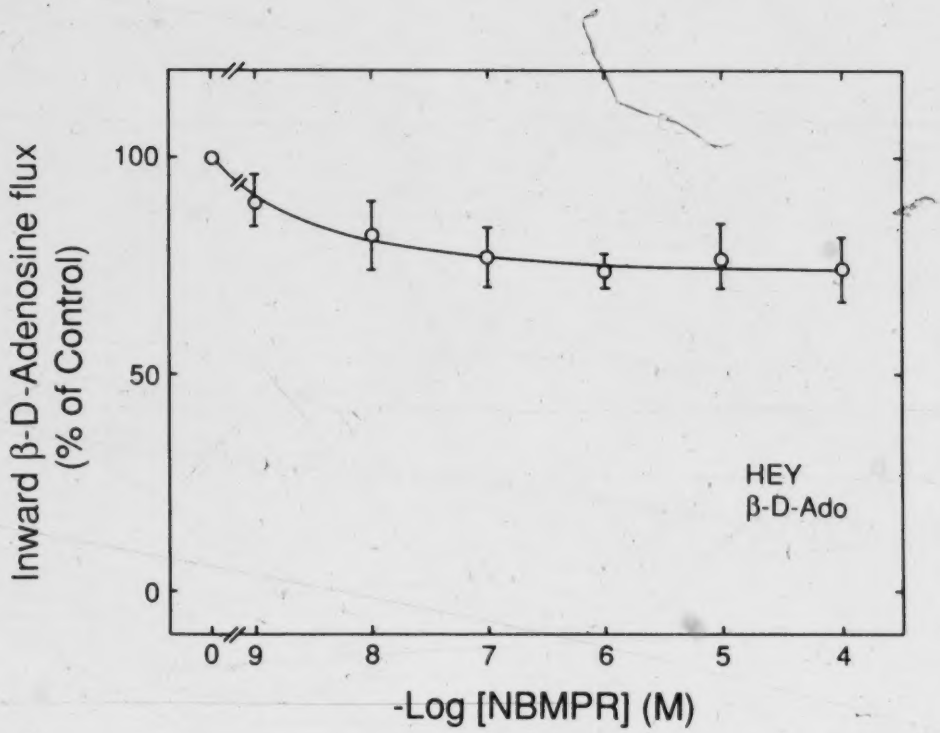

Fig. 31. Low sensitivity of Ado fluxes in HEY cells to NBMPR inhibition. Replicate cultures were incubated at 22° for 15 min with or without graded NBMPR concentrations prior to assays of Ado influx. Intervals of uptake were started by flooding monolayers with transport medium containing ^{3}H-Ado with and without graded concentrations of NBMPR, and ended by immersion of dishes in ice-cold PBS. Fluxes are expressed as percentages of those in the absence of NBMPR (3.1 ± 0.5 pmol/10^{6} cells/sec, 100%). Data are means ± S.E. from 3 experiments, each with assays in triplicate.

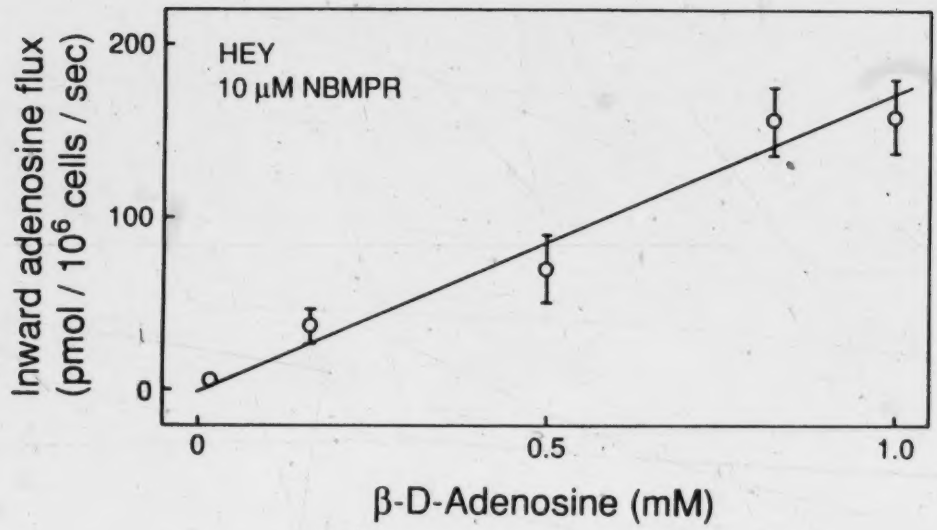

Fig. 32. Concentration dependence of Ado fluxes in HEY cells. Time courses of ^{3}H-Ado influx in replicate HEY cultures were determined in NBMPR-containing medium as described in Fig. 18. Ado fluxes were determined from such time course data and plotted against permeant concentration. Values shown are means ± S.E. from 3 similar experiments, each employing triplicate assays.

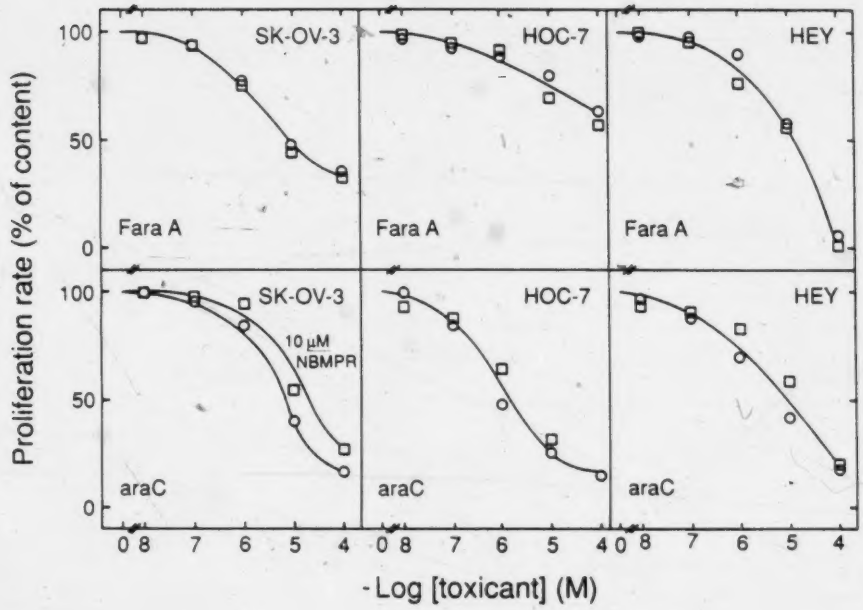

Fig. 33. Failure of NBMPR to protect ovarian carcinoma cells in culture against the growth inhibitory effects of araC and FaraA. Monolayers were cultured in medium containing graded concentrations of FaraA (upper panels) and araC (lower panels), with (□) or without (○) 10 μM NBMPR. Proliferation rates (number of cell population doublings during 72hr) in the presence of these agents, are expressed as percentages of rates in the absence of drug (3.1 for SK-OV-3, 4.2 for HOC-7 and 5.1 for HEY). Means of triplicate assays are plotted; similar results were obtained when the experiment was repeated.

V. DISCUSSION

A. Experimental Chemotherapy of Murine Tumours

Host Protection Studies

Biochemical modulation, the utilisation of one or more agents (which might be devoid of cytotoxic activity) to enhance the cytotoxicity or selectivity of antineoplastic drugs, has been a topic of interest in recent years in attempts to overcome the clinical problems of tumour resistance and host toxicity of drugs.

Protection of rodents against otherwise lethal dosages of nucleoside analogues with NT inhibitors has been reported from this laboratory (Lynch *et al.*, 1981a,b; Paterson *et al.*, 1983; Jakobs and Paterson, 1984). In those studies, tubercidin and nebularine were the most frequently utilised toxicants, and NBMPR-P, a pro-drug form of NBMPR, was used as a protectant. Several potent NT inhibitors have been identified in recent years, and various cytotoxic nucleoside analogues with promising antineoplastic activity have become available. The present study investigated the "host-protection" approach to chemotherapy with combinations of several NT inhibitors and cytotoxic nucleoside analogues.

The NT inhibitors used in the present study differed in effectiveness in the protection of mice against the nucleoside lethality. Surprisingly, the rank order of their potencies (LD_{50} values) did not correlate with the rank order of their *in vitro* abilities to inhibit adenosine transport in S49 mouse lymphoma cells (Paterson *et al.*, 1982). The rank order of protectant potency also varied with the route of inhibitor administration (Figs. 2 and 3) and the toxic nucleoside. When the NT inhibitors were administered subcutaneously, NBdAdo-P appeared to be the most potent protectant against the lethality of tubercidin and nebularine (Fig. 2). The difference in potency between NBdAdo-P and NBMPR-P, however, was not statistically significant ($p > 0.05$). Dilazep and NBMPR-P were the most potent protectants against toxicant lethality among the intraperitoneally-administered inhibitors (Fig. 3). The effectiveness of NT inhibitors at protecting animals against the toxicity of toxic nucleoside

analogues depends on the analogue chosen. This is illustrated by 5-azacytidine. In contrast to other inhibitor-toxicant combinations studied, co-administration of NBTGR-P enhanced the toxicity of 5-azacytidine, whereas treatment with NBdAdo-P and NBMPR-P had no apparent effect on the toxicity of that agent (Fig. 4 and Table 8). Enhancement of nucleoside analogue toxicity in the following analogue-NT inhibitor combinations has been reported: pyrazofurin-NBMPR-P, pyrazofurin-dilazep, pseudoisocytidine-NBMPR-P and 5-fluorouridine-dilazep (Paterson *et al.*, 1983a). Such enhancement may be due to inhibitor-induced retention of drug in dose-limiting host tissues (which may differ from one drug to another) through blockade of efflux processes by the NT inhibitors.

The different potencies of the NT inhibitors in protecting mice against nucleoside lethality (LD_{50} values), and the large influence of administration route on potency, probably reflect differences in inhibitor pharmacokinetics. For instance, dipyridamole, at doses that were the molar equivalent of protective doses of other NT inhibitors, was found to be a poor protectant against nucleoside analogue toxicity in these studies. Dipyridamole in the circulation mainly (99%) in the form of the plasma protein bound agent (Mahony *et al.*, 1982; Kopitar *et al.*, 1971), suggesting that the concentration of free dipyridamole in the dose-limiting host tissues may be substantially lower than the apparent concentration in the circulation. Also, when administered intraperitoneally, NBdAdo (7 mg/kg, qd x 5) was a more potent protectant in mice against nebularine toxicity than dilazep (40 mg/kg, qd x 5) (Paterson *et al.*, 1983b). In the present study, intraperitoneally-administered NBdAdo-P (50 mg/kg, qd x 5), the soluble pro-drug of NBdAdo, was a less potent protectant of mice against nebularine toxicity (Fig. 3 and Table 6) than was intraperitoneally-administered dilazep (50 mg/kg, qd x 5). Perhaps, differences in rates of dephosphorylation *in vivo* of the 5'-monophosphoesters of NBMPR, NBTGR and NBdAdo, the pro-drug forms of these agents, may contribute to the observed differences in their potency in host protection against toxic nucleoside analogues.

Chemotherapy

Several reports describe the ip administration of host-protective doses of NT inhibitors in combination with potentially lethal doses of cytotoxic nucleosides in the treatment of rodents bearing neoplasms, pathogenic protozoa such as trypanosomes or plasmodia, or schistosomes (El Kouni *et al.*, 1983, 1987; Gati *et al.*, 1987; Jakobs and Paterson, 1986; Ogbunude and Ikediobi, 1982). Evidently, the NT inhibitors reduced the access of the toxicants to drug-sensitive host tissues, without blocking access to cells that are the therapeutic targets.

In the present study, sc treatment of mice implanted with neoplastic cells with NBMPR-P, prior to ip treatment of the same animals with toxic nucleoside analogues, enhanced tumour cell kill and increased life span relative to the effects achieved by treatment with tubercidin or nebularine alone ($p < 0.05$). As may be seen in Tables 2a, 2b and 2c, none of the leukaemic mice treated with tubercidin or nebularine alone was cured, reflecting the inherent lack of activity of these two drugs against leukaemias P388 and L1210. Cures of mice implanted with the Ehrlich ascites carcinoma, a very immunogenic neoplasm, were achieved.

Subcutaneously-administered NBdAdo-P and NBMPR-P were the most potent protectants against the lethality of toxic nucleoside analogues, but chemotherapy experiments in which mice with L1210 leukaemia were treated with sc NBdAdo-P and ip tubercidin combinations yielded results which were inferior to similar experiments using NBMPR-P (results not shown). Similarly, when the inhibitors and toxicants were administered intraperitoneally, dilazep and NBMPR-P proved to be the most effective protectants. Chemotherapy with the dilazep-nebularine combination (results not shown), however, yielded results that were similar to treatment with nebularine-NBMPR-P combinations (Lynch *et al.*, 1981a). The superiority of NBMPR-P in these chemotherapy experiments might be due to the contributory antineoplastic effect of a 6-thiopurine metabolite. Paterson and co-workers have presented data supporting this idea (Cass *et al.*, 1974; Lynch *et al.*, 1981a).

The findings of this study suggest that the host protection tactic has considerable promise in improving the therapeutic indices of cytotoxic nucleosides. *In vitro* NT inhibitor

Table 11. Treatment of $B6D2F_1$ mice implanted with 10^6 leukaemia L1210/C2 cells with combinations of FAMP and NBMPR-P.[1]

FAMP[2,3] (mg/kg)	NBMPR-P[3] (mg/kg)	ILS[4] (%)	60-Day survivors	Paralysis
200	0	125	11/49	11/49
200	25	200	30/50	0/50
225	0	131	2/20	4/20
225	25	194	12/20	1/20
250	0	125	3/20	6/20
250	25	163	8/20	0/20

[1] Modified from Adjei, A. A., Dagnino, L. D., Wong, M., and Paterson, A. R. P., manuscript in preparation.

[2] 2-fluoro-9-β-D-arabinosyladenine 5'-monophosphate.

[3] Treatment protocol: ip (qd, days 1-5)

[4] Increase in life span (see Table 3a).

potencies are poor predictors of chemotherapeutic effectiveness of inhibitor-toxicant combinations, since pharmacokinetic properties and possible synergistic antineoplastic effects of inhibitor metabolites might contribute to therapeutic effects. It may be possible to develop rationales for combining NT inhibitors and nucleoside drugs in the treatment of particular neoplasms. For instance, in a recent study (Table 11), $B6D2F_1$ mice implanted with leukaemia L1210 cells were treated with fludarabine phosphate (FAMP) and NBMPR-P administered by the ip route as individual agents or in combinations. Combination of NBMPR-P and FAMP resulted in substantial enhancement of leukaemic cell kill relative to that achieved with either agent alone. This enhancement may be due to NBMPR-induced retention of FAMP metabolites in L1210 cells. Such effects have been shown in L1210 cells with other nucleoside analogues (Dagnino *et al.*, 1987) and appear to be attributable to the properties of the NT systems expressed in L1210 cells (Dagnino, 1988). Also apparent in the Table 11 study of the antileukaemia activity of FAMP-NBMPR-P combinations was a fatal neurotoxicity of FAMP, initially evident in leukaemic mice as hind-limb paralysis. NBMPR-P protected mice against this neurotoxicity.

B. Nucleoside Transport in Ovarian Carcinoma Cells

As an initial step in extending the host-protection tactic to the treatment of human neoplastic disease, we undertook the characterisation of nucleoside permeation system(s) in human ovarian carcinoma cells. This neoplasm is typically confined to the peritoneal cavity during most of its natural history and, therefore, is a candidate for intracavitary chemotherapy strategies.

Nucleoside Metabolism

The principal initial metabolic fates of adenosine molecules entering cells are phosphorylation to nucleotides and deamination to form inosine and with subsequent phosphorolysis to hypoxanthine (Savarese *et al.*, 1983).

In HOC-7 cells, intracellular adenosine was rapidly phosphorylated (about 50% in 30 sec. Fig. 26). Little deamination occured under the latter conditions, even at high adenosine concentrations. In most animal cells, Ado phosphorylation occurs at low adenosine concentrations (below 5 μM) and Ado deamination is favoured at high concentrations because adenosine kinase has a significantly lower K_m than adenosine deaminase (Schrader *et al.*, 1972). Because HOC-7 cells appeared to have little adenosine deaminase activity, HOC-7 experiments were conducted in the absence of adenosine deaminase inhibitors.

Virtually no metabolism of either β-L-Ado or formycin B occured during one hour of incubation at 22°C with HOC-7 cells. The absence of β-L-Ado metabolites in HOC-7 cells demonstrated the expected stereospecificity of the adenosine-metabolising enzymes in these cells. Formycin B has been shown to be poorly metabolised in animal cells (Paterson *et al.*, 1987; Jakobs and Paterson, 1986; Dagnino, 1988); the similar finding in this study provided the basis for the use of formycin B in loading HOC-7 cells for countertransport experiments.

Adenosine Transport

As a consequence of the rapidity of nucleoside transport in animal cells, it has been necessary to develop rapid sampling methods in order to to define the time courses of nucleoside permeation during the first few seconds of cell exposure to permeant. The methods developed include (i) "instantaneous" stoppage of nucleoside fluxes by inhibitors such as NBMPR and dilazep in cells with inhibitor-sensitive transport systems, and (ii) rapid centrifugation of cells through an oil layer to isolate them from permeant-containing medium by using fast-starting microcentrifuges (Paterson *et al.*, 1985). These methods are employed with cells in suspension. With monolayers, cells on cover slips (Hawkins and Berlin, 1969; Taube and Berlin, 1972), in culture dishes (Heichal *et al.*, 1978; Jakobs and Paterson, 1986), and in bottles (Paterson *et al.*, 1977a,b) may be exposed to medium containing labelled permeant for brief intervals ended by flooding (bottles) or by dipping drained monolayers in cold medium. In the present study, a minor modification of this method allowed measurement of permeant influx during intervals as short as 2 seconds. Time-zero values of adenosine

uptake, the measurement of which was described in Materials and Methods, were approximately proportional to permeant concentration, suggesting that physical processes such as adsorption might account for these values.

Inward Ado fluxes in SK-OV-3 cells were saturable (Fig. 8), indicating that the influx process was transporter-mediated; the K_m value for this process at 22°C was about 39 μM. Inward Ado fluxes in HOC-7 cells had a major, non-saturable component (Fig. 16). A Hanes-Woolf plot of those data indicated that two permeation processes contributed to Ado fluxes (Fig. 17), a saturable process with a K_m of 28 μM and a non-saturable process. The K_m values for Ado transport in these two cell lines are in general agreement with the reported values of < 42 μM in nucleated cells (22°) from this laboratory (Paterson *et al.*, 1985) and by Fox and Kelley (1978). In contrast, values reported by Plagemann and co-workers are 2- to 5-fold higher (Plagemann and Wohlhueter, 1980; Plagemann *et al.*, 1988). This discrepancy may have a methodological basis. In the former reports, kinetic parameters have been calculated from time course data in experiments that measure time-zero uptakes of permeants, while in the latter reports, data from time courses of permeant uptake have been fitted to integrated rate equations from which initial rates were calculated without experimental determination of time-zero permeant uptakes.

Inhibition of Adenosine Transport

Facilitated diffusion NT systems have been conveniently classified by sensitivity to NBMPR inhibition. In this laboratory, NT systems of "high" and "low" NBMPR sensitivity are usually designated as those with IC_{50} values for NBMPR inhibition of < 10 nM or > 1 μM, respectively. Table 12 compares such IC_{50} values for some cultured animal cell types. In HOC-7 cells, NBMPR concentrations of 20 μM inhibited Ado fluxes by only 30% and concentration-effect relationships for inhibition of Ado fluxes by the NT inhibitors dilazep or dipyridamole were identical to that for NBMPR. These results are different from those observed in many other cell types, in which dipyridamole will effect complete NT inhibition

Table 12. NBMPR inhibition of nucleoside transport in cultured animal cells.

Cell line	IC_{50} (μM)
3924A (rat hepatoma)	1[1]
Walker 256 (rat carcinosarcoma)	10[2]
S49 (mouse lymphoma)	0.001[2]
SK-OV-3 (human ovarian carcinoma)	0.005[7]
HOC-7 (human ovarian carcinoma)	IND[3],[7]
HEY (human ovarian carcinoma)	IND[3],[7]
NUB-6 (human neuroblastoma)	0.01[4],[5]
HeLa (human cervical carcinoma)	0.05[6]
CI 80-13S (human ovarian carcinoma)	0.004[8]

[1] Ng, 1986

[2] Gati *et al.*, 1986

[3] Indeterminate. At NBMPR concentrations of 20 μM, inhibition was less than 50%.

[4] Kaplinsky *et al.*, 1986

[5] Nucleoside permeant was thymidine. Adenosine was used in the other studies.

[6] Cass and Paterson, 1977

[7] Present study.

[8] Jamieson *et al.*, 1989, studied the permeation of araC.

whereas dilazep and NBMPR effect partial inhibition (Jarvis and Young, 1986; Kubota *et al.*, 1988; Woffendin and Plagemann, 1987). These findings indicate that HOC-7 cells express Ado permeation processes with properties different from those previously described in animal cell types with NT systems of low NBMPR sensitivity.

Ado fluxes in SK-OV-3 cells were more sensitive to NBMPR inhibition than in HOC-7 cells, in that such fluxes reduced by about 80% in the presence of 10 μM NBMPR. These results are consistent with observations in other human neoplastic cell lines (HeLa, NUB-6, HL-60, CI 80-13S) which express components of nucleoside permeation processes that are of low NBMPR sensitivity (Jamieson *et al.*, 1989; Kaplinsky *et al.*, 1986; Kubota *et al.*, 1988; Paterson *et al.*, 1977a).

Site-specific NBMPR Binding to Ovarian Carcinoma Cells

NBMPR has been a very useful probe in study of the function and biology of NT systems in animal cells. High affinity NBMPR binding sites are thought to represent NT elements in the plasma membrane. Supporting evidence includes the demonstration by Cass *et al.*, (1974) that fractional inhibition of uridine transport in human erythrocytes was proportional to fractional occupancy of NBMPR binding sites. As well, cells of the AE_1 clone of S49 mouse lymphoma possessed no NBMPR binding sites, and were virtually devoid of NT capability (Cass *et al.*, 1981).

In contrast, Novikoff UA cells (Gati *et al.*, 1986) and rat hepatoma 3924A cells (Ng, 1986) possess high affinity NBMPR binding sites which appear to be "uncoupled" from the NT system. In spite of this, NBMPR binding sites are generally assumed to signify the presence of facilitated diffusion NT elements in the cell membrane, although the apparent complexity in the interaction between NBMPR binding and facilitated diffusion transporters in nucleated cells should not be ignored. High affinity NBMPR binding sites are present on both HOC-7 and SK-OV-3 cells under equilibrium conditions (Figs. 5 and 12). Mass law analysis of the binding data yielded K_D values for NBMPR dissociation from the

Table 13. Comparison of NBMPR binding constants for some human neoplastic cell lines.

Cell line	K_D nM	Bmax sites/cell
HOC-7 (ovary)[1]	2.9	9×10^5
SK-OV-3 (ovary)[1]	2.2	4×10^5
HL-60 (leukaemia)[2]	2.3	2.4×10^5
IMR-32 (neuroblastoma)[3]	-	1.2×10^5
RPMI 6410 (leukaemia)[4]	0.48	3.3×10^5
K562 (leukaemia)[5]	0.23	5×10^5
CI 80-13S (ovary)[6]	1.35	2.3×10^5

[1] binding constants reported for these cell lines are means from 6 experiments.

[2] Chen *et al.*, 1986

[3] Kaplinsky *et al.*, 1986

[4] Paterson *et al.*, 1979b

[5] Boleti, H., and Cass, C. E., unpublished results.

[6] Jamieson *et al.*, 1989.

NBMPR-binding site complex of 2.9 ± 0.4 nM for HOC-7, which was similar to the SK-OV-3 value of 2.2 ± 0.4 nM (Figs. 4 and 11). The NBMPR site density was also of the same order of magnitude, 9×10^5 and 4×10^5 sites/cell for HOC-7 and SK-OV-3 cells respectively. These values are in general agreement with those of other cultured human neoplastic cells in the literature (Table 13). By these criteria, HOC-7 cells would appear to possess a facilitated diffusion NT mechanism, similar to that of SK-OV-3 cells.

Transporter-Independent Permeation of Adenosine in HOC-7 Cells

Ado fluxes in erythrocytes of sheep (Young, 1978), guinea pigs (Roos and Pfleger, 1972) and humans (Turnheim *et al.*, 1978) as well as in erythrocyte ghosts (Schrader *et al.*, 1972) have been reported to have a diffusional component. In some of these studies, long time courses were utilised for influx measurement and the rates reported reflect the tandem processes of transport and metabolism, rather than transport only.

The component of low NBMPR sensitivity in Ado fluxes observed in HOC-7 cells (Fig. 14) was recognised as a transporter-independent route of Ado permeation by the kinetic characteristics of that process, that is, by the direct proportionality between flux and substrate concentrations of up to 1 mM (Fig. 19). In that study, transporter-independent Ado fluxes were measured in the presence of 10 μM NBMPR, a concentration that blocks facilitated diffusion Ado fluxes in many cell types. Validity of the flux-concentration proportionality as a criterion of transporter-independent permeation requires that fluxes be measured over a wide range of substrate concentrations because fluxes of permeants with high K_m values would show linear flux-concentration relationships at low substrate concentrations (Wilbrandt, 1975). Because K_m values for Ado fluxes in nucleated animal cells between 5 and 200 μM have been reported (see earlier sections), and because plasma adenosine concentrations are usually less than 1 μM (Sollevi *et al.*, 1987), we considered that the maximum Ado concentration of 1 mM would be appropriate for exploration of flux-concentration relationships in HOC-7 cells.

β-L-Adenosine Permeation

Transporters, like enzymes, exhibit enantiomeric substrate selectivity (Hofer, 1977). Thus, the naturally occuring isomer, D-glucose, is transported at rates which are at least ten-fold greater than its enantiomer, L-glucose, in intact cells as well as in vesicles. L-glucose has been used in glucose permeation studies to measure rates of glucose entry by simple diffusion (Carter *et al.*, 1972; Turner and Moran, 1982a,b; Barnett *et al.*, 1973).

Work in this laboratory has shown that the three NT systems presently recognised in animal cells, the faciltated diffusion systems of high and low NBMPR sensitivity, and the concentrative, sodium-linked system, are highly stereoselective. These systems distinguish between the D- and L-enantiomers ("mirror-image isomers") of Ado (Fig. 34). In the experiments summarised in Table 14, inward fluxes β-L-Ado were not significantly different from zero in non-ovarian cell lines and ten-fold lower than β-D-Ado fluxes in SK-OV-3 and rat hepatic cells. Thus, β-L-Ado fluxes would appear to approximate rates of diffusional entry of β-D-Ado in these cell types. In the present study, β-L-Ado was used to characterise the substantial, transporter-independent Ado permeation process in HOC-7 cells. Influx of 15 μM β-L-Ado in HOC-7 cells was rapid, non-concentrative and not inhibited by exposure of the cells to medium containing 10 μM NBMPR (Fig. 22). This influx approximated that of β-D-Ado 10 μM NBMPR (Fig. 30). Inward β-L-Ado fluxes in HOC-7 cells were strictly proportional to concentration over a wide range of concentrations (Fig. 25) and the fluxes measured were not inhibited by thousand-fold molar excesses of either β-D-Ado or 2'-deoxyadenosine (Fig. 23). These findings indicate that entry of β-L-Ado into HOC-7 cells is a transporter-independent process; the magnitude of that process in HOC-7 cells is remarkable.

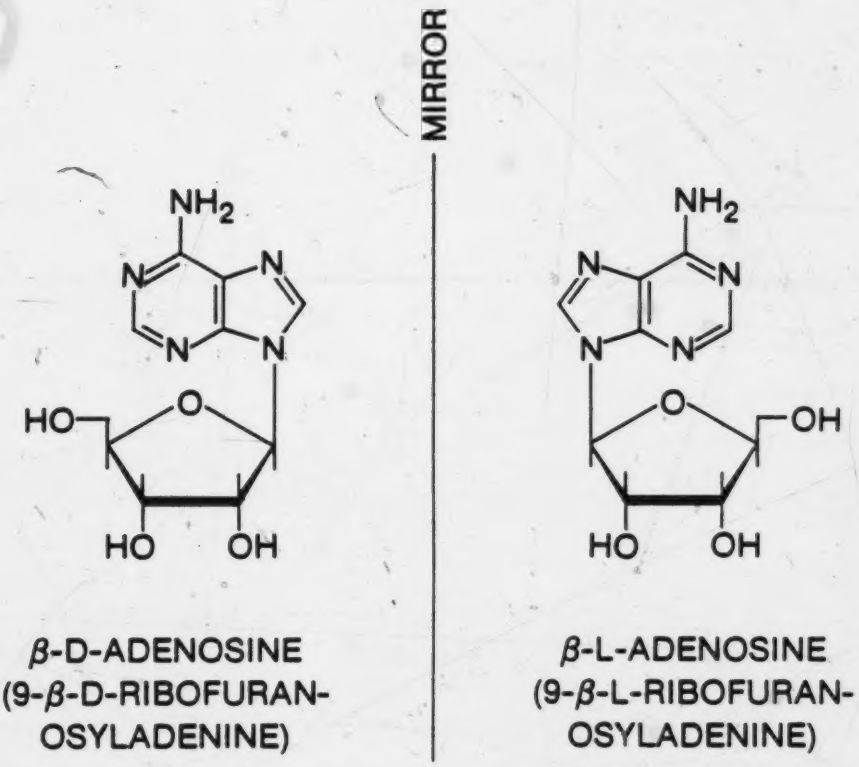

Fig. 34. Enantiomers of adenosine.

Table 14. Comparison of β-D-Ado and β-L-Ado fluxes in some animal cells.

Cell type	β-D-Ado fluxes	β-L-Ado fluxes	Permeant concentration (μM)
IEC-6[1] rat enterocytes	2pm/10^6 cells/sec	0	5
L1210 [2]	0.22pm/μl cell H_2O/sec	0	10
rat hepatocytes[3]	4pm/μl cell H_2O/sec	0.4	50
SK-OV-3 human ovarian carcinoma	7pm/10^6 cells/sec	0.8	15
HOC-1 human ovarian carcinoma	5pm/10^6 cells/sec	4.2	15
HOC-7 human ovarian carcinoma	6.5pm/10^6 cells/sec	4	15
HEY human ovarian carcinoma	3.9pm/10^6 cells/sec	3	15

[1] Jakobs, E.S. unpublished results

[2] Dagnino, 1988

[3] Awumey, E.M.K. unpublished results.

Plasmodium yoelii-infected mouse erythrocytes express "new" parasite-induced pathways of Ado permeation. The native nucleoside transporter of these cells catalyses a facilitated diffusion process of high NBMPR sensitivity. One of the two parasite-induced processes appears to be non-saturable, of low NBMPR-sensitivity, and of low enantiomeric selectivity in that β-L-Ado is a permeant. In contrast to the transporter-independent permeation route in HOC-7 cells, β-L-Ado fluxes through the parasite-induced pathway are inhibited by furosemide (Gati *et al.*, manuscript in preparation).

In exploring mechanisms for the passage of two related compounds across the plasma membrane, demonstrations of countertransport with the two permeants constitute important evidence that the same carrier or transporter mediates the transmembrane passage of both permeants (Stein, 1986; Rosenberg and Wilbrandt, 1957; Widdas, 1952; Hofer, 1977). Formycin B and β-D-Ado were countertransport permeants in HOC-7 cells, while β-L-Ado was not so accepted (Fig. 27). These results agree with the interpretation that β-L-Ado permeation in these cells is a transporter-independent process. However, because of the joint expression of transporter-independent and facilitated diffusion processes in HOC-7 cells, counterflow transport processes with formycin B would not be expected to occur. The results obtained therefore argue that formycin B may not be a permeant for the transporter-independent Ado permeation pathway.

Temperature - dependence of Adenosine Transport

Transporter-mediated fluxes exhibit a strong temperature-dependence, increasing two- to three-fold for a 10° increase in temperature (Jain, 1988). Diffusional fluxes show a less marked temperature-dependence. This difference has been used as a criterion for distinguishing between the two processes (Zimmerman *et al.*, 1987; Hofer, 1977; Berlin, 1973; Schafer and Barfuss, 1986).

However, while minimal temperature -dependence may identify diffusional fluxes, the converse is not necessarily true. For instance the diffusion of L-glucose into Novikoff rat hepatoma cells (Graff *et al.*, 1977) and the permeation of water into the human neutrophil

(West, 1983) were found to have temperature dependences that were greater than some transporter-mediated systems.

Fluxes of β-L-Ado and β-D-Ado in HOC-7 cells in the presence of NBMPR were essentially unaffected by increasing temperature (Fig. 21). The lack of temperature-dependence (within experimental error) in the permeation of Ado enantiomers in HOC-7 cells argues further for the expression of a transporter-independent, Ado permeation process in HOC-7 cells..

Transporter-independent Nucleoside Fluxes in HOC-7 Cells

Diffusional fluxes of solutes across animal cell membranes involve partitioning of the substance into the membrane, its diffusion within, and partitioning out of the membrane (Davson and Danielli, 1952; Lieb and Stein, 1971). This process is a function of the solute's partition coefficient (describing its solubility in the membrane), its diffusion coefficient (describing its rate of movement within the membrane), and the thickness of the membrane (Stein, 1986; Graff *et al.*, 1977). These points illustrate the importance of membrane properties as determining factors in diffusional fluxes, and explain why such fluxes differ with cell type. Reports of transporter-independent nucleoside fluxes in animal cells have shown the operating mechanism to be that of simple diffusion across the lipid bilayer, and that rates of permeation correlate with the lipid-buffer partition coefficients of the permeants (Roos and Pfleger, 1972; Domin *et al.*, 1988; Zimmerman *et al.*, 1987).

We have investigated flux-concentration relationships for the permeation of several physiological nucleosides in HOC-7 cells in the presence of 10 μM NBMPR, a condition under which the facilitated diffusion NT system in HOC-7 cells is blocked, permitting exploration of the transporter-independent pathway. Such flux-concentration relationships were linear (Fig. 28) and yielded rate constants that describe the transporter-independent permeation rates. The permeation rate constants so obtained did not correlate with the lipophilicities of the nucleoside permeants (Table 10 and Fig. 29). Furthermore, physiological nucleosides are relatively hydrophilic molecules, and their diffusional fluxes across most animal cell

membranes will be slow (Plagemann and Wohlhueter, 1980; Schafer and Barfuss, 1986; Stein, 1986).

The rapid fluxes of adenosine and other nucleosides through the transporter-independent pathway, the lack of correlation between these fluxes and permeant lipophilicities, and the virtual absence of temperature-dependence, are consistent with the presence of a transporter-independent, "channel-like" route of nucleoside influx in HOC-7 cells. Trypan blue, which is able to enter some viable, lysolecithin-permeabilised tumour cells such as BHK monolayer cells (Miller *et al.*, 1979), was excluded from HOC-7 cells, as was sucrose, thus ruling out the possibility of flux contributions by non-specific leaks of permeants. The physiological significance and origins of this nucleoside permeation mechanism are unknown.

Attempts to inhibit permeation through this pathway with protein modifiers and agents such as phloretin and furosemide which are known to inhibit transporter-independent permeation pathways in several animal cells (Ginsburg and Stein, 1987a; Bashford *et al.*, 1988), were unsuccessful.

Nucleoside Permeation in HOC-1 and HEY Cells

HOC-1 ovarian carcinoma cells were established in culture from ascitic fluid from a patient, who, eight months later, was the donor of another sample of ascitic fluid from which the HOC-7 cell line was established. HOC-1 cells were permeable to β-L-Ado and fluxes of the latter agent were of low sensitivity to NBMPR, indicating the operation of a transporter-independent nucleoside permeation process similar to that described in HOC-7 cells. Thus, this permeation pathway was expressed in that patient's ovarian carcinoma at the time of the sampling which yielded the HOC-1 cell line.

Cells of the HEY ovarian carcinoma line also expressed the NBMPR-insensitive permeability to β-L-Ado, as well as low sensitivity of β-D-Ado influx to NBMPR inhibition. The flux-concentration relationship for β-D-Ado permeation in the presence of NBMPR was linear (Fig. 32). These results showed the presence of a transporter-independent ,

channel-like route of nucleoside permeation similar to that described for HOC-7 cells. This permeation process may well be expressed in other ovarian carcinoma cells and possibly some other human neoplasms. If true, that possibility has important implications for chemotherapy with cytotoxic nucleoside and NT inhibitor combinations.

In Vitro Toxicity Assays

Nucleoside analogues commonly inhibit the growth of many lines of neoplastic cells in culture, and cultured cells with NBMPR-sensitive NT systems may be protected by NBMPR and other NT inhibitors against the cytotoxic effects of nucleoside analogues (Cass *et al.*, 1981; Paterson *et al.*, 1979b). Conversely, neoplastic cells with significant components of low NBMPR sensitivity in their nucleoside permeation processes have not been protected against the antiproliferative effects of toxic nucleoside analogues by NT inhibitors (Kaplinsky *et al.*, 1986; Kubota *et al.*, 1988). The failure of NBMPR to protect neoplastic cells against cytotoxic nucleosides *in vitro* has been a good predictor of therapeutic benefit of treatment regimens employing the host-protection strategy concerned (Ng, 1986; Jakobs and Paterson, 1984). We have demonstrated the lack of *in vitro* NBMPR protection against the toxicity of FaraA and araC in three human ovarian carcinoma cells in culture. With HEY and HOC-7 cells, these findings are consistent with the contribution of a major, transporter-independent process to nucleoside fluxes in these cells. Results with SK-OV-3 cultures suggest that an NBMPR-insensitive process which is 20% of nucleoside fluxes is large enough to allow the influx of lethal amounts of toxic nucleosides, which would make chemotherapy measures based on the host-protection idea feasible in such cell types.[17] These findings support the prediction that combinations of tumour-sensitive cytotoxic nucleosides and NBMPR would be effective in the treatment of human tumours with similar nucleoside permeation characteristics.

[17]Dr. Judith Belt has made similar findings in a number of cultured human rhabdomyosarcoma cell lines (personal communication).

C. Conclusions and Future Directions

Combinations of cytotoxic nucleosides and NT inhibitors would appear to provide a means of increasing the therapeutic index of cytotoxic nucleosides. This strategy could be guided rationally by preliminary investigations that would select specific inhibitor - toxicant combinations for well-characterised neoplastic cell types. NBMPR appears to be the most potent NT inhibitor for chemotherapy purposes, and phase I trials of this drug are warranted.

A major component of transporter-independent nucleoside permeation has been identified in HOC-7 and HEY cells, two cultured lines of human ovarian adenocarcinoma cells. SK-OV-3, another human ovarian adenocarcinoma cell line, did not express the transporter-independent permeation pathway. This pathway appears to be a channel-like or pore-like route, that excludes sucrose, and is neither enantioselective nor NBMPR-sensitive. This process appears to have low temperature-dependence and inhibitors are not known. The "adenosine channel" would allow two approaches to chemotherapy: (i) selective delivery of cytotoxic nucleosides to cells expressing this route by blocking entry into host tissues with NT inhibitors, and, (ii) selective entry into the target cells of toxic nucleosides that are poor substrates for NT systems. The first approach would also be appropriate for cells such as SK-OV-3 that possess a component of the conventional NBMPR-insensitive NT system. Because HEY and SK-OV-3 cells have been adapted to grow ip as xenografts in immune-deprived mice (Baumal *et al.*, 1986; Ward *et al.*, 1987), these strategies may be tested *in vivo*.

Since the potential for selectively attacking tumour cells that express the transporter-independent, channel-like permeation route of nucleosides is great, a survey of available human cell lines should be made to determine if this activity is commonly expressed.

Finally, the molecular biology of this channel-like permeation route must be explored, to answer questions pertaining to selectivity and operating mechanism(s). As well, attempts should be made to isolate the "channel" protein.

REFERENCES

Adjei, A. A., and Paterson, A. R. P. (1988). Non-mediated permeation of adenosine (ado) in a human ovarian carcinoma cell line. **Proc. Am. Ass. Cancer Res. 29:** 14.

Agbanyo, F. R., Cass, C. E., and Paterson, A. R. P. (1988). External location of sites on pig erythrocyte membranes that bind nitrobenzylthioinosine. **Mol. Pharmacol. 33:** 332-337.

Almeida, A. F., Jarvis, S. M., Young, J. D., and Paterson, A. R. P. (1984). Photoaffinity labelling of nucleoside transporter of cultured mouse lymphoma cells. **FEBS Lett. 176:** 444-448.

Angielski, S., Le Hir, M., and Dubach, U. C. (1983). Transport of adenosine by renal brush border membranes. **Pflugers Arch. 397:** 75-77.

Asai, M., Hieda, H., and Shimizu, B. (1967). Studies on synthetic nucleotides: Preparation of L-β-ribonucleosides and L-β-ribonucleotides. **Chem. Pharm. Bull. 15:** 1863-1870.

Awumey, E. M. K. (1989). Pyrimidine nucleoside transport in isolated rat hepatocytes. **M. Sc. Thesis.** University of Alberta.

Baba, T., and Nishikawa, K. (1980). Effective combination of anticancer drug with its antidote for chemotherapy of hepatic metastasis. **Gann. 71:** 157-158.

Barber, H. R. K. (1986). Ovarian cancer. **Ca-A Cancer Journal for clinicians. 36:** 149-189.

Barnett, J. E. G., Holman, G. D., and Munday, K. A. (1973). Structural requirements for

binding to the sugar transport system of the human erythrocyte. **Biochem. J. 131:** 211-221.

Bashford, C. L., Alder, G. M., Graham, J. M., Menestrina, G., and Pasternak, C. A. (1988). Ion modulation of membrane permeability: Effect of cations on intact cells and on cells and phospholipid bilayers treated with pore forming agents. **J. Memb. Biol. 103:** 79-94.

Bashford, C. L., Micklem, K. J., and Pasternak, C. A. (1985). Sequential onset of permeability changes in mouse ascites cells induced by Sendai virus. **Biochem. Biophys. Acta 814:** 247-255.

Bech-Hansen, N. T., Till, J. E., and Ling, V. (1975). Pleiotropic phenotype of colchicine-resistant CHO cells: Cross-resistance and colateral sensitivity. **J. Cell. Physiol. 88:** 23-32.

Baumal, R., Law, J., Buick, R. N., Kahn, H., Yeger, H., Sheldon, K., Colgan, T., and Marks, A. (1986). Monoclonal antibodies to an epithelial ovarian adenocarcinoma: Distinctive reactivity with xenografts of the original tumor and a cultured cell line. **Cancer Res. 46:** 3994-4000.

Belt, J. A.(1983). Heterogeneity of nucleoside transport in mammalian cells. Two types of transport activity in L1210 and other neoplastic cells. **Mol. Pharmacol. 24:** 479-488.

Belt, J.A. and Noel, L.D.(1985). Nucleoside transport in Walker 256 carcinosarcoma and S49 mouse lymphoma cells. **Biochem. J. 232:** 681-688.

Berlin, R. D. (1973). Temperature dependence of nucleoside membrane transport in rabbit

alveolar macrophages and polymorphonuclear leukocytes. **J. Biol. Chem. 248:** 4724-4730.

Berlin, R. D., and Oliver, J. M. (1975). Membrane transport of purine and pyrimidine bases and nucleosides in animal cells. **Int. Rev. Cytol. 42:** 287-336

Bhakdi, S., and Tranum-Jensen, J. (1984). Mechanism of complement cytolysis and the concept of channel-forming proteins. **Phil. Trans. R. Soc. Lond. B306:** 311-324.

Bhuyan, B. K., Scheidt, L. G., and Fraser, T. J., (1972). Cell cycle phase specificity of antitumor agents. **Cancer Res. 32:** 398-407.

Bibi, O., Schwartz, J., Eilam, Y., and Cabantchik, Z. I. (1978). Nucleoside transport in mammalian cell membranes iv. Organomercurials and organomercurial-mercaptonucleoside complexes as probes for nucleoside transport systems in hamster cells. **J. Membrane Biol. 39:** 159-183.

Biesele, J. J., Staughterback, M. C., and Margolis, M., (1955). Unsubstituted purine and its riboside as toxic antimetabolites in mouse tissue cultures. **Cancer 8:** 87-96.

Bisel, H. F., Ansfield, F. J., Mason, J. H., and Wilson, W. L., (1970). Clinical studies with tubercidin administered by direct intravenous injection. **Cancer Res. 30:** 76-78.

Bols, N.C., Bowen, B.W.M., Khor, K.G. and Boliska, S.A. (1980). Thin-layer chromatography of thymidine and thymidine metabolites in enzyme assays and cell extracts. **Anal. Biochem. 106:** 230-237.

Bowman, R. J. and Levitt, D. G. (1977). Polyol permeability of the human red cell. Interpretation of glucose transport in terms of a pore. **Biochim. Biophys. Acta. 466:**

68-83.

Brown, G. B., and Weliky, V. S. (1953). The synthesis of 9-β-D-ribofuranosylpurine and the identity of nebularine. **J. Biol. Chem. 204**: 1019-1024

Buick, R. N., Pullano, R., and Trent, J. M., (1985). Comparative properties of five human ovarian adenocarcinoma cell lines. **Cancer Res. 45**: 3668-3676.

Cabantchik, Z. I., and Ginsburg, H. (1977).Transport of uridine in human red blood cells. Demonstration of a simple carrier-mediated process. **J. Gen. Physiol. 69**: 75-96

Cain, J. M., Saigo, P. E., Pierce, V. K., Clark, D. G., Jones, W. B., Smith, D. H., Hakes, T. B., Ochoa, M. and Lewis, J. L. (1986). A review of second-look laporotomy for ovarian cancer. **Gynecol. Oncol. 23**: 14-25

Camiener, G.W. and Smith, C.G.(1965). Studies on enzymatic deamination of cytosine arabinoside. **Biochem. Pharmacol. 14**: 1405-1416.

Carter, J. R., Avruch, J. and Martin, D. B. (1972). Glucose transport in plasma membrane vesicles from rat adipose tissue. **J. Biol. Chem. 247**: 2682-2688

Cass, C. E., Belt, J. A. and Paterson, A. R. P. (1987). Adenosine transport in cultured cells and erythrocytes. **Prog. in Clin. and Biol. Res. 230**: 13-40.

Cass, C.E., Gaudette, L.A. and Paterson, A.R.P. (1974). Mediated transport of nucleosides in human erythrocytes. Specific binding of the inhibitor nitrobenzylthioinosine to nucleoside transport sites in the erythrocyte membrane. **Biochim. Biophys. Acta 345**: 1-10.

Cass, C. E., and Janowska-Wieczorek, A. (1988). Modulation of tubercidin uptake in human hematopoietic cells by inhibitors of nucleoside transport. **Proc. Am. Ass. Cancer Res. 29:** 14.

Cass, C.E., Kolassa, N., Uehara, Y., Dahlig-Harley, E.R. and Paterson, A.R.P. (1981). Absence of binding sites for the transport inhibitor nitrobenzylthioinosine on nucleoside transport-deficient mouse lymphoma cells. **Biochim. Biophys. Acta 649:** 769-777.

Cass, C. E., and Paterson, A. R. P. (1972). Mediated transport of nucleosides in human erythrocytes. Accelerative exchange diffusion of uridine and thymidine and specificity toward pyrimidine nucleosides as permeants. **J. Biol. Chem. 247:** 3314-3320

Cass, C. E., and Paterson, A. R. P. (1975). Inhibition by nitrobenzylthioinosine of uptake of adenosine, 2'-deoxyadenosine, and 9-β-D-arabinofuranosyladenine by human and mouse erythrocytes. **Biochem. Pharmacol. 24:** 1989-1993

Cass, C. E. and Paterson, A. R. P. (1976). Nitrobenzylthioinosine binding sites in the erythrocyte membrane. **Biochim. Biophys. Acta 419:** 769-777.

Cass, C. E., and Paterson, A. R. P. (1977). Inhibition of thymidine uptake in asynchronous HeLa cells by nitrobenzylthioinosine. **Exp. Cell Res. 105:** 427-435

Chello, P. L., Sirotnak, F. M., Dovick, D. M., Yang, C-H., and Montgomery J. A., (1983) Initial rate kinetics and evidence for duality of mediated transport of adenosine, related purine nucleosides, and nucleoside analogues in L1210 cells. **Cancer Res. 43:** 97-103.

Chen, S-F., Cleaveland, J. S., Hollman, A. B., Wiemann, R. F., Parks, R. E. Jr., and Stoeckler, J. D. (1986). Changes in nucleoside transport of HL-60 human promyelocytic

cells during N,N-Dimethylformamide induced differentiation. **Cancer Res. 46:** 3449-3455.

Cohen, A. Ullman, B. and Martin, D.W. (1979). Characterization of a mutant mouse lymphoma cell with deficient transport of purine and pyrimidine nucleosides. **J. Biol. Chem. 254:** 112-116.

Cohen, A., Leung, C. and Thompson, E. (1985). Characterization of mouse lymphoma cells with altered nucleoside transport. **J. Cell Physiol. 123:** 431-434.

Cohen, S., (1966). Introduction to the biochemistry of D-arabinosyl nucleosides. **Progress In Nucleic Acid Res. 5:** 1-88.

Crowther, P. J., Cooper, I. A., and Woodcock, D. M. (1985). Biology of cell killing by 1-β-D-arabinofuranosylcytosine and its relevance to molecular mechanisms of cytotoxicity. **Cancer Res. 45:** 4291-4300.

D'Acquisto, R., Markman, M., Hakes, T., Rubin, S., Hoskins, W., and Lewis, J. L. (1988). A phase I trial of intraperitoneal recombinant Gamma-interferon in advanced ovarian carcinoma. **J. Clin. Oncol. 6:** 689-695.

Dagnino, L., (1988) Sodium-linked nucleoside transport in mouse leukemia L1210 cells. **PhD Thesis.** University of Alberta.

Dagnino, L., Bennett, L.L. Jnr. and Paterson, A.R.P.(1987). Concentrative transport of nucleosides in L1210 mouse leukemia cells. **Proc. Am. Assoc. Cancer Res. 28:** 15.

Dahlig-Harley, E., Eilam, Y., Paterson, A.R.P. and Cass, C.E.(1981). Binding of nitrobenzylthioinosine to high-affinity sites on the nucleoside transport mechanism of

HeLa cells. **Biochem. J. 200:** 295-305.

Danielli, J. F. (1954). Morphological and molecular aspects of active transport. **Symp. Soc. Exp. Biol. 8:** 502-516.

Davson, H. and Danielli, J. F. (1952). The effect of temperature on permeability. In : **The permeability of natural membranes.** Cambridge university press. pp 244-248.

Domin, B. A., Mahony, W. B. and Zimmerman, T. P. (1988). 2',3'-dideoxythymidine permeation of the human erythrocyte membrane by nonfacilitated diffusion. **Biochem. Biophys. Res. Comm. 154:** 825-831.

Drach, J. C., and Novack, J. M. (1973). Some simple thin-layer chromatographic systems for the separation of purines and purine nucleosides. **Anal. Biochem. 52:** 633-636.

Dulbecco, R., and Voght, M. (1974). Plaque formation and isolation of pure lines with poliomyelitis viruses. **J. Exp. Med. 99:** 167-182

Edsall, J. T., and Wyman, J. (1958). **Biophysical Chemistry**, Academic Press, New York

Eilam, Y., and Cabantchik, Z. I. (1977). Nucleoside transport in mammalian cell membranes: a specific inhibitory mechanism of high affinity probes. **J. Cell. Physiol. 92:** 185-202.

El Kouni, M. H., Diop, D., and Cha, S. (1983) Combinaton therapy of schistosomiasis by tubercidin and nitrobenzylthioinosine 5'-monophosphate. **Proc. Natl. Acad. Sci. 80:** 6667-6670.

El Kouni, M. H., Messier, N. J., and Cha, S. (1987). Treatment of schistosomiasis by purine nucleoside analogues in combination with nucleoside transport inhibitors. **Biochem. Pharmacol. 36:** 3815-3821.

Ellory, J. C., Elford, B. C. and Newbold, C. I. (1988). Transport mechanisms across cell membranes. **Parasitology 96:** S5-S9.

Finean, J. B., Coleman, R. and Michell, R. H. (1984). **Membranes and their cellular functions.** Blackwell scientific publications. Oxford, London, Edinburgh, Boston, Melbourne. pp 49-76.

Fogh, J., and Trempe, G. (1975). New human tumor cell lines. In: **Human tumor cells in vitro** (Fogh, J. ed.) Plenum Press, New York. pp 115-159

Fogh, J., Wright, W. C., and Loveless, J. D. (1977). Absence of HeLa cell contamination in 169 cell lines derived from human tumors. **J. Natl. Cancer Inst. 58:** 209-214

Fonken, G. (1970) Development of ara-C. In: **Proceedings of the chemotherapy conference on ara-C : development and application.** pp 165-180. (Carter, S. K. and Livingston, R. B. eds) USPHS 1970.

Fox, I. H., and Kelley, W. N. (1978). The role of adenosine and 2'-deoxyadenosine in mammalian cells. **Ann. Rev. Biochem. 47:** 655-686.

Frei, E., Bickers, J. N., Hewlett, J. S., Lane, M., Leary, W. V., and Talley, R. W. (1969) Dose schedule and antitumor studies of arabinosyl cytosine (NSC 63878). **Cancer Res 29:** 1325-1332.

Furth, J., and Cohen, S. (1968) Inhibition of mammalian DNA polymerase by the 5'-triphosphate of 1-β-D-arabinofuranosylcytosine and the 5'-triphosphate of 9-β-D-arabinofuranosyladenine. **Cancer Res. 28:** 2061-2067.

Gati, W. P., Belt, J. A., Jakobs, E. S., Young, J. D., Jarvis, S. M., and Paterson, A. R. P. (1986). Photoaffinity labelling of a nitrobenzylthioinosine-binding polypeptide from cultured Novikoff hepatoma cells. **Biochem. J. 236:** 665-670.

Gati, W. P., and Paterson, A. R. P. (1989). Nucleoside transport. In: **The red cell membrane: structure, function, and clinical implications.** (Agre, P., and Parker, J. C., eds). Marcel Dekker, New York. pp 635-661.

Gati, W. P., Stoyke, A. F-W., Gero, A. M. and Paterson, A. R. P. (1987). Nucleoside permeation in mouse erythrocytes infected with *plasmodium yoelii*. **Biochem. Biophys. Res. Comm. 145:** 1134-1141.

Gero A. M., Bugledich, E. M. A., Paterson, A. R. P., and Jamieson, G. P. (1988). Stage-specific alteration of nucleoside membrane permeability and nitrobenzylthioinosine insensitivity in *Plasmodium falciparum* infected erythrocytes. **Mol. Biochem. Parasitol. 27:** 159-170.

Ginsburg, H. and Stein, W. D. (1987a), Biophysical analysis of novel transport pathways induced in red blood cell membranes. **J. Memb. Biol. 96:** 1-10.

Ginsburg, H. and Stein, W. D. (1987b). New permeability pathways induced by the malarial parasite in the membrane of its host erythrocyte: Potential routes for targeting of drugs into infected cells. **Bioscience Reports 7:** 455-463.

Ginsburg, H., Kutner, S., Krugliak, M. and Cabantchik, Z. I. (1985). Characterisation of permeation pathways appearing in the host membrane of plasmodium falciparum infected red blood cells. **Mol. and Biochem. Parasit. 14:** 313-322.

Goldenberg, G. J. and Begleiter, A. (1984). Alterations of drug transport. **In: Antitumor drug resistance.** (Fox, B. W. and Fox, M. eds) Springer-Verlag. Berlin, Heidelberg, New York, Tokyo. pp 241-298.

Graff, J. C., Wohlhueter, R. M. and Plagemann, P. G. W. (1977). Effect of temperature and of cytochalasin B and persantin on the nonmediated permeation of non-electrolytes into cultured Novikoff rat hepatoma cells. **J. Biol. Chem. 252:** 4185-4190.

Grage, T. B., Rochlin, D. B., Weiss, A. J. and Wilson, W. L. (1970). Clinical studies with tubercidin administered after absorption into human erythrocytes. **Cancer Res. 30:** 79-81.

Graham, F., and Whitmore, F. (1970). Studies in mouse L-cells on the incorporation of 1-β-D-arabinofuranosylcytosine 5'-triphosphate. **Cancer Res. 30:** 2636-2644.

Hahn, F. E. (ed.) (1979). Mechanism of action of antieukaryotic and antiviral compounds. In: **Antibiotics** Vol. 5, Part 2. Springer-Verlag, Berlin.

Hamburger, A. W., Dunn, F. E., and White, C. P. (1984). Separation of cells derived from human malignant effusions on percoll density gradients. **Proc. Am. Ass. Cancer Res. 25:** 29.

Hammond, J. R., Paterson, A. R. P., and Clanachan, A. S. (1981). Benzodiazepine inhibition of site-specific binding of nitrobenzylthioinosine, an inhibitor of adenosine transport. **Life Sci. 29:** 2207-2214

Harley, E. R., Paterson, A. R. P., and Cass, C. E. (1982). Initial rate kinetics of the transport of adenosine and 4-amino-7-(β-D-ribofuranosyl)pyrrolo[2,3-d]pyrimidine (tubercidin) in cultured cells. **Cancer Res. 42:** 1289-1285

Hawkins, R. A., and Berlin, R. D. (1969). Purine transport of nucleosides in rabbit polymorphonuclear leukocytes. **Biochim. Biophys. Acta 255:** 6-18.

Heichal, O., Bibi, O., Katz, J., and Cabantchik, Z. I. (1978). Nucleoside transport in mammaliam cell membranes. Kinetic and chemical modification studies of cytosine-arabinoside and uridine transport in hamster cells in culture. J. **Membrane. Biol. 39:** 133-157

Heichal, O., Ish-Shalom, D., Koren, R. and Stein, W. D. (1979). The kinetic dissection of transport from metabolic trapping during substrate uptake by intact cells: Uridine uptake by quiescent and serum-activated Nil 8 hamster cells and their MSV-transformed counterparts. **Biochem. Biophys. Acta 551:** 169-186.

Hofer, M. (1977). **Transport Across Biological Membranes**. Translated by J. G. Hoggett (1981). Pitman, Boston, London, New York.

Howell, S. B., Chu, B. B. F., Wung, W. E., Metha, B. M., and Mendelsohn, J. (1981). Long duration intracavitary infusion of methotrexate with systemic leukovorin protection in patients with malignant effusions. J. **Clin. Invest. 67:** 1161-1170.

Howell, S. B., Pfeiffle, C. L., Wung, W. E., Olshen, R. A. Lucas, W. E., Yon, J. L., and Green, M. (1982) Intraperitoneal cisplatin with systemic thiosulphate protection. **Ann. Int. Med. 97:** 845-851.

Howell, S. B., Pfeiffle, C. E., Wung, W. E., and Olshen, R. A. (1983). Intraperitoneal cisplatin with systemic thiosulfate protection. **Cancer Res. 43:** 1426-1431

Irish, C. E., Awrich, A., Moseson, D., Davenport, C., Mosely, H. S., and Fletcher, W. S. (1979) 5-fluorouracil, tubercidin, streptozotocin and adriamycin : effective agents in the management of functioning and advanced islet cell tumors of the pancreas. **Proc. Am. Assoc. Cancer Res. 20:** 361.

Iwamoto, Y., Kawano, T., Uozumi, J., Aoki, K., and Baba, T. (1984). "Two-route chemotherapy" using high-dose ip cisplatin and iv sodium thiosulfate, its antidote, for peritoneally disseminated cancer in mice. **Cancer Treat. Rep. 68:** 1367-1373.

Jain, M. K. (1988). **Introduction to Biological Membranes.** John Wiley & Sons. New York, Chichester, Brisbane, Toronto. pp 257-280.

Jakobs, E. S., and Paterson, A. R. P. (1984). Treatment of the Walker 256 carcinosarcoma (W256) with tubercidin and nitrobenzylthioinosine 5'-monophosphate. **Proc. Am. Assoc. Cancer Res. 25:** 381.

Jakobs, E. S. and Paterson, A. R. P. (1986). Sodium-dependent, concentrative nucleoside transport in cultured intestinal epithelial cells. **Biochem. Biophys. Res. Comm. 140:** 1028-1035.

Jamieson, G. P., Snook, M. B., Bradley, T. R., Bertoncello, I., and Wiley, J. S. (1989). Transport and metabolism of 1-β-D-Arabinofuranosylcytosine in human ovarian adenocarcinoma cells. **Cancer Res. 49:** 309-313.

Jarvis, S. M. (1986). Nitrobenzylthioinosine-sensitive nucleoside transport system: mechanism

of inhibition by dipyridamole. **Mol. Pharmacol. 30:** 659-665.

Jarvis, S. M., Ellory, J. C., and Young, J. D. (1986). Radiation inactivation of the human erythrocyte nucleoside and glucose transporters. **Biochim. Biophys. Acta. 855:** 312-315.

Jarvis, S. M., Fincham, D. A., Ellory, J. C., Paterson, A. R. P., and Young, J. D. (1984). Nucleoside transport in human erythrocytes. Nitrobenzylthioinosine binding and uridine transport activities have similar radiation target site. **Biochim. Biophys. Acta 772:** 227-230

Jarvis, S. M., Hammond, J. R., Paterson, A. R. P., and Clanachan, A. S. (1982a) Species differences in nucleoside transport.A study of uridine transport and nitrobenzylthioinosine binding by mammalian erythrocytes. **Biochem. J. 208:** 83-88

Jarvis, S. M., Janmohamed, S. N., and Young, J. D. (1983). Kinetics of nitrobenzylthioinosine binding to the human erythrocyte nucleoside transporter. **Biochem. J. 216:** 661-667

Jarvis, S. M. and Martin, B. W. (1986). Effects of temperature on the transport of nucleosides in guinea pig erythrocytes. **Can. J. Physiol. Pharmacol. 64:** 193-198.

Jarvis, S.M. and Young, J.D. (1978). Genetic control of nucleoside transport in sheep erythrocytes. **Biochem. Genetics 16:** 1035-1043.

Jarvis, S. M., McBride, D., and Young, J. D. (1982b). Erythrocyte nucleoside transport: assymetrical binding of nitrobenzylthioinosine to nucleoside permeation sites. **J. Physiol. (Lond) 324:** 31-46

Jarvis, S. M., and Young, J. D. (1980). Nucleoside transport in human and sheep

erythrocytes. Evidence that nitrobenzylthioinosine binds specifically to functional nucleoside-transport sites. **Biochem. J. 190:** 377-383

Jarvis, S. M., and Young, J. D. (1982a). Nucleoside translocation in sheep reticulocytes and fetal erythrocytes: A proposed model for the nucleoside transporter. J. **Physiol. 324:** 47-66

Jarvis, S. M., and Young, J. D. (1982b). Inhibitor binding studies.Nitrobenzylthioinosine, a specific high-affinity inhibitor of nucleoside transport. In: **Red Cell Membranes - A Methodological Approach** (Ellory, J. C., and Young, J. D. ed.) Academic Press, London. pp. 263-272.

Jarvis, S. M., Young, J. D., and Ellory, J. C. (1980). Nucleoside transport in human erythrocytes. Apparent molecular weight of the nitrobenzylthioinosine-binding complex estimated by radiation-inactivation analysis. **Biochem. J. 190:** 373-376

Jarvis, S. M. and Young, J. D. (1986). Nucleoside transport in rat erythrocytes : two components with differences in sensitivity to inhibition by nitrobenzylthioinosine and p-mercuriphenylsulfonate. **J. Membrane Biol. 93:** 1-10.

Jarvis, S. M., and Young, J. D. (1987). Photoaffinity labelling of nucleoside transporter polypeptides. **Pharmac. Ther. 32:** 339-359.

Janmohamed, S. N., Young, J. D., and Jarvis , S. M. (1985). Proteolytic cleavage of [^{3}H]nitrobenzylthioinosine-labelled nucleoside transporter in human erythrocytes. **Biochem. J. 230:** 777-784.

Johnston, M. E., and Geiger, J. D. (1989). Sodium-dependent uptake of nucleosides by

dissociated brain cells from the rat. **J. Neurochem. 52:** 75-81.

Jones, R. B., Collins, J. M., Myers, C. E., Brooks, A. E., Hubbard, S. M., Balow, J. E., Brennan, M. F., Dedrick, R. L., and DeVita, V. T. (1981). High-volume intraperitoneal chemotherapy with methotrexate in patients with cancer. **Cancer Res. 41:** 55-59

Kaplinsky, C., Yegar, H., Estrov, Z., Barankiewicz, J., Pawlin, G., Freedman, M. V., and Cohen, A. (1986) Selective protection of tubercidin toxicity by nitrobenzylthioinosine in normal tissues but not in human neuroblastoma cells. **Cancer Chemother. Pharmacol. 17:** 264-268.

Khym, J. X. (1975). An analytical system for rapid separation of tissue nucleotides at low pressures on conventional anion exchangers. **Clin. Chem. 21:** 1245-1252.

King, M. E., Naporn, A., Young, B., and Howell, S. B. (1980). Modulation of cytarabine uptake and toxicity by dipyridamole. **Cancer Treat. Rep. 68:** 361-366.

King, M. E., Pfeiffle, C. E., and Howell, S. B. (1984). Intraperitoneal cytosine arabinoside therapy in ovarian carcinoma. **J. Clin. Oncol. 2:** 662-669.

Klip, A., Walker, D., Cohen, A., and Leung, C. (1986). Chemical and genetic comparison of the glucose and nucleoside transporters. **Biochem. Cell Biol. 64:** 1170-1180.

Kolassa, N., and Pfleger, K. (1975). Adenosine uptake by erythrocytes of man, rat and guinea pig and its inhibition by hexobendine and dipyridamole. **Biochem. Pharmacol. 24:** 154-156

Kolassa, N., Stengg, R. and Turnheim, K. (1978). Influence of hexobendine, dipyridamole,

dilazep, lidoflazine, inosine and purine riboside on adenosine uptake by the isolated epithelium of guinea pig jejunum. **Pharmacol. 16:** 54-60.

Kopitar, Z., and Weizenberger, H. (1971). Specific binding of dipyridamole to human serum protein; its isolation, identification, and characterisation as alpha-1 acidic glycoprotein. **Arzneim. Forsch. 21:** 859-862.

Koren, R., Shohami, E. and Yeroushalmi, S. (1979). A kinetic analysis of the uptake of cytosine-β-D-arabinoside by rat B77 cells. Differentiation between transport and phosphorylation. **Eur. J. Biochem. 95:** 333-339.

Koren, R., Cass, C. E., and Paterson, A. R. P. (1983). The kinetics of dissociation of the inhibitor of nucleoside transport, nitrobenzylthioinosine from the high-affinity binding sites of cultured hamster cells. **Biochem. J. 216:** 299-308

Krakoff, I. (1987). Cancer chemotherapeutic agents. **Ca-A Cancer Journal for Clinicians. 37:** 93-105.

Kubota, M., Takimoto, T., Kitoh, T., Tanizawa, A., Kiriyama, Y., Akiyama, Y. and Mikawa, H. (1988). Prevention of 1-Beta-D-arabinofuranosylcytosine toxicity by 4-nitrobenzyl-6-thioinosine or dipyridamole in human leukaemia cell lines. **Anticancer Res. 8:** 339-342.

Kufe, D. W., and Major, P. P. (1982). Studies on the mechanism of action of cytosine arabinoside. **Med. Pediatr. Oncol. 1:** 49-67 (suppl)

Kufe, D. W., and Spriggs, D. R. (1985). Biochemical and cellular pharmacology of cytosine arabinoside. **Seminars in Oncology 12:** 34-48.

Kutner, S., Breuer, H., Ginsburg, H., and Cabantchik, Z. I. (1985). Characterisation of permeation pathways in the plasma membrane of human erythrocytes infected with early stages of *Plasmodium falciparum*: Association with parasite development. **J. Cell. Physiol. 125:** 521-527

Kutner, S., Ginsburg, H., and Cabantchik, Z. I. (1983). Permselectivity changes in malaria (*Plasmodium falciparum*) infected human blood cell membranes. **J. Cell. Physiol. 114:** 245-251

Kwan, K. F., and Jarvis, S. M. (1984). Photoaffinity labelling of adenosine transporters in cardiac membranes with nitrobenzylthioinosine. **Am. J. Physiol. 246:** 710-715.

Le Hir, M., and Dubach, U. C. (1984). Sodium gradient-energized concentrative transport of adenosine in renal brush border vesicles. **Pflugers Arch. 401:** 58-63

Le Hir, M., and Dubach, U. C. (1985). Uphill transport of pyrimidine nucleosides in renal brush border vesicles. **Pflugers Arch. 404:** 238-243

Leo, A., Hansch, C., and Elkins, D. (1971). Partition coefficients and their uses. **Chem. Rev. 71:** 525-616.

Leyland-Jones, B. and O'Dwyer, P. J. (1986). Biochemical modulation: Application of laboratory models to the clinic. **Cancer Treat. Rep. 70:** 219-229.

Lieb, W. R. (1982). A kinetic approach to transport studies. In: **Red Cell Membranes - A Methodological Approach.** Ellory, J. C. and Young, J. D., (eds). Academic Press, London. pp. 135-164

Lieb, W. R. and Stein, W. D. (1971). The molecular basis of simple diffusion within biological membranes. **Curr. Topics Memb. Transport 2:** 1-39.

Lieb, W. R., and Stein, W. D. (1974). Testing and characterizing the simple carrier. **Biochim. Biophys. Acta 373:** 178-196

Lindbergh, B., Klenow, H., and Hansen, K. (1967). Some properties of partially purified mammalian adenosine kinase. J. **Biol. Chem. 242:** 350-356.

Lum, C. T., Marz, R., Plagemann, P. G. W., and Wohlhueter, R. M. (1979). Adenosine transport and metabolism in mouse leukemia cells and in canine thymocytes and peripheral blood leukocytes. J. **Cell. Physiol. 101**: 173-200

Lynch, T. P., Naik, S. R., Cass, C. E., and Paterson, A. R. P. (1978). Inhibition of nucleoside uptake in HeLa cells by nitrobenzylthioinisinate. **Biochem. Pharmacol. 27:** 1303-1304

Lynch, T. P., Jakobs, E. S., Paran, J. H., and Paterson, A. R. P. (1981a). Treatment of mouse neoplasms with high doses of tubercidin. **Cancer Res. 41:** 3200-3204

Lynch, T. P., Paran, J. H., and Paterson, A. R. P. (1981b). Therapy of mouse leukemia L1210 with combinations of nebularine and nitrobenzylthioinosine 5'-monophosphate. **Cancer Res. 41:** 560-565

Mahony, C., Wolfram, K. M., Coccheto, D. M., Bjornsson, T. D. (1982). Dipyridamole kinetics. **Clin. Pharmacol. Ther. 31:** 330-338.

Mahony, W. B., and Zimmerman, T. P. (1986). An assay for inhibitors of nucleoside

transport based upon the use of 5-[^{125}I]iodo-2'-deoxyuridine as permeant. **Anal. Biochem. 154:** 235-243.

Maiden, M. C. J., Davis, E. D., Baldwin, S. A., Moore, D. C. M. and Henderson, P. J. F. (1987). Mammalian and bacterial sugar transport proteins are homologous. **Nature (Lond) 325:** 641-643.

Maier, C., Bremer, E., Schmid, A. and Benz, R. (1988). Pore-forming activity of the Tsx protein from the outer membrane of *Escherichia coli*. **J. Biol. Chem. 263:** 2493-2499.

Major, P. P., Egan, E. M., Beardsley, G. P., Minden, M. D., and Kufe, D. W. (1981). Lethality of human myeloblasts correlates with the incorporation of arabinofuranosyl cytosine into DNA. **Proc. Natl. Acad. Sci. 78:** 3235-3239.

Mantovani, A., Allavena, P., Sessa, C., Bolis, G., and Mangioni, C. (1980). Natural killer activity of lymphoid cells isolated from human ascitic ovarian tumours. **Int. J. Cancer, 25:** 573-582.

Markman, M., and Howell, S. B. (1985). Intraperitoneal chemotherapy for ovarian carcinoma. In: **Ovarian Cancer.** (Alberts, D. S., and Surwit, E. A. eds) Martinus Nijhoff Publishers. Boston. pp 179-212.

Markman, M. and Howell, S. B. (1987). Intraperitoneal chemotherapy: Principles and results of clinical trials. In: **Concepts, clinical developments, and therapeutic advances in cancer chemotherapy. (Muggia, F. M. ed.)** Martinus Nijhoff. Boston. pp 39-62.

Mayrand, R. R., and Levitt, D. G. (1983). Urea and ethylene glycol-facilitated transport systems in the human red cell membrane. **J. Gen. Physiol. 81:** 221-237.

Miller, M. R:, Castellot, J. J. and Arthur, B. P. (1979). A general method for permeabilizing monolayer and suspension cultured animal cells. **Exp. Cell Res. 120**: 421-423.

Minami, R., Yokota, S., and Teplitz, R. L. (1978). Gradient separation of normal and malignant cells II. Application to in vivo tumor diagnosis. **Acta Cytol. 22**: 584-588.

Muggia, F. M., and Carter, S. K. (1987). Malignant diseases. In: **Avery's Drug Treatment. Principles and Practice of Clinical Pharmacology and Therapeutics.** (Speight, T. M., ed) Adis Press, Auckland. pp 1023-1077.

Muller, W. E. G. (1979). Uptake and metabolism of purine nucleosides and purine nucleoside analogues by cells. In: **Nucleoside Analogues** (Walker, R.T., DeClercq, E., and Eckstein, F., eds) Plenum Press, New York, pp. 247-279

Naccache, P., and Sha'afi, R. I. (1973). Patterns of nonelectrolyte permeability in human red blood cell membrane. **J. Gen. Physiol. 62**: 714-736.

Nakae, T. (1976). Outer membrane of salmonella. Isolation of protein complex that produces transmembrane channels. **J. Biol. Chem. 251**: 2176-2178.

Nakamura, G. (1961). Studies on antibiotic actinomycetes III. On streptomyces producing 9-β-D-Ribofuranosylpurine. **J. Antibiotics (Tokyo) 14A**: 94-97.

National Cancer Institute. (1984) **In vivo cancer models**. NIH Publication No. 84-2635 Bethesda, Md.

National Cancer Institute. (1988). **NCI Investigational Drugs**. NIH Publication No. 89-2141. Bethesda, Md.

Neame, K.D. and Richards, T.G. (1972). Analysis of experimental data. In: **Elementary Kinetics of Membrane Carrier Transport**. Blackwell Scientific Publications, Oxford, London, pp. 41-55.

Ng, C.Y.C. (1986). Nucleoside transport systems of low sensitivity to nitrobenzylthioinosine in cultured rat hepatoma cells. **M.Sc. Thesis**, University of Alberta.

Nikaido, H. and Rosenberg, E. Y. (1981). Effect of solute size on diffusion rates through the transmembrane pores of the outer membrane of *Escherichia coli*. **J. Gen. Physiol. 77**: 121-135.

Nikaido, H., and Vaara, M. (1985). Molecular basis of bacterial outer membrane permeability. **Microb. Rev. 49**: 1-32.

Noel, W. C., and Robins, R. K. (1962). The antitumor activity of 2-amino-6-alkylthio-9-β-D-ribofuranosylpurines and related derivatives of 2-amino-6-purinethiol (thioguanine). **J. Med. Pharm. Chem. 5**: 1074-1085

Oberg, B., and Johansson, N.-G. (1984). The regulative merits and drawbacks of new nucleoside analogues with clinical potential. **J. Antimicrob. Chemother. 14: Suppl. A**,pp. 5-26

O'Dwyer, P. J., Leyland-Jones, B., and Hoth, D. F. (1987). The clinical horizon of deoxycoformycin. In: **Concepts, Clinical developments and therapeutic advances in Cancer chemotherapy**. (Muggia, F. M., ed) Martinus Nijhoff Boston, Lancaster. pp 233-255.

Ogbunude, P. O. J., Gati, W. P., and Paterson, A. R. P. (1984). Dephosphorylation of nitrobenzylthioinosine 5'-monophosphate by ecto 5'-nucleotidase of HeLa cells. **Biochem.**

Pharm. 33: 3561-3566

Ogbunude, P. O. J. and Ikediobi, C. O. (1982). Effect of nitrobenzylthioinosinate on the toxicity of tubercidin and ethidium against *trypanosoma gambiense*. **Acta Tropica 39:** 219-224.

Oliver, J. M., and Paterson, A. R. P. (1971). Nucleoside transport. A mediated process in human erythrocytes. **Can. J. Biochem. 49:** 262-270

Owen, S. P., and Smith, C. G. (1964). Cytotoxicity and antitumor properties of the abnormal nucleoside tubercidin (NSC- 56408). **Cancer Chemother. Rep. 36:** 19.

Ozols, R. F., and Young, R. C. (1987). Ovarian cancer. **Current Problems in Cancer Res. 11:** 57-122.

Pande, S. V. (1976). Liquid scintillation counting of aqueous samples using triton-containing scintillants. **Anal. Biochem. 74:** 25-34

Parks, R. E., and Brown, P. R. (1973). Incorporation of nucleosides into the nulceotide pools of human erythrocytes.Adenosine and its analogs. **Biochemistry 12:** 3294-3302

Paterson, A. R. P., and Cass, C. E. (1986). Transport of nucleoside drugs in animal cells. In: **International Encyclopaedia of Pharmacology and Therapeutics** (I. D. Goldman, ed). Pergamon Press, Oxford. pp. 309-329.

Paterson, A. R. P., Harley, E. R., and Cass, C. E. (1984). Inward fluxes of adenosine in erythrocytes and cultured cells measured by a quenched-flow method. **Biochem. J. 224:** 1001-1008

Paterson, A. R. P., Harley, E. R., and Cass, C. E. (1985). Measurement and inhibition of membrane transport of adenosine. In: **Methods in Pharmacology 6:** 165-180 (Paton, D. M. ed) Plenum Publishing Corp.

Paterson, A. R. P., Harley, E. R., Kolassa, N., and Cass, C. E. (1981a). Transport of nucleosides in animal cells. In: **Nucleosides and Cancer Treatment** (M.H.N. Tattersall and Fox, R.M., eds) Academic Press, Sydney, Australia. pp. 3-17

Paterson, A. R. P., Kolassa, N., and Cass, C. E. (1981b). Transport of nucleoside drugs in animal cells. **Pharmac. Ther. 12:** 515-536

Paterson, A. R. P., Jakobs, E. S., Harley, E. R., Cass, C. E., and Robins, M. J. (1983a). Inhibitors of Nucleoside Transport as Probes and Drugs. In: **Development of Target-Oriented Anticancer Drugs** (Cheng, Y.-C., Goz, B., and Minkoff, M., eds) Raven Press, New York, pp. 41-56

Paterson, A. R. P., Jakobs, E. S., Harley, E. R., Fu, N., Robins, M. J., and Cass, C. E. (1983b). Inhibition of nucleoside transport. In: **Regulatory Function of Adenosine** (Berne, R.M., Rall, T.W., and Rubio, R. eds) Martinus Nijhoff Publishers, The Hague, pp. 203-220

Paterson, A. R. P., Lau, E. Y., Dahlig, E., and Cass, C. E. (1980). A common basis for inhibition of nucleoside transport by dipyridamole and nitrobenzylthioinosine? **Mol. Pharmacol. 18:** 40-44

Paterson, A. R. P., Naik, S. R., and Cass, C. E. (1977a). Inhibition of uridine uptake in HeLa cells by nitrobenzylthioinosine and related compounds. **Mol. Pharmacol. 13:** 1014-1023

Paterson, A. R. P., Babb, L. R., Paran, J. H., and Cass, C. E., (1977b). Inhibition by nitrobenzylthioinosine of adenosine uptake by asynchronous HeLa cells. **Mol. Pharmacol. 13**: 1147-1158.

Paterson, A. R. P., Paran, J. H., Yang, S., and Lynch, T. P., (1979a). Protection of mice against lethal dosages of nebularine by nitrobenzylthioinosine, an inhibitor of nucleoside transport. **Cancer Res. 39**: 3607-3611

Paterson, A. R. P., Yang, E. S-E., Lau, E. Y., and Cass, C. E. (1979b). Low specificity of the nucleoside transport mechanisn of RPMI 6410 cells. **Mol. Pharmacol. 16**: 900-908

Paterson, A.R.P., Jakobs, E.S., Ng, C.Y.C., Odegard, R.D., and Adjei, A.A. (1987). Nucleoside transport inhibtion *in vitro* and *in vivo*. In: **"Topics and Perspectives in Adenosine Research."** Ed. by: E. Gerlach, B.F. Becker. Springer-Verlag Berlin Heidelberg, 1987. pp. 89-101.

Paul, B., Chen, M. F., and Paterson, A. R. P. (1975). Inhibitors of nucleoside transport. A structure-activity study using human erythrocytes. **J. Med. Chem. 18**: 968-973

Pickard, M. A., and Paterson, A. R. P. (1972). Use of 4-nitrobenzylthioinosine in the measurement of rates of nucleoside transport in human erythrocytes. **Can. J. Biochem. 233**: 688-701

Plagemann, P. G. W., and Kraupp, M. (1986). Inhibition of nucleoside and nucleobase transport and nitrobenzylthioinosine binding by dilazep and hexobendine. **Biochem. Pharmacol. 35**: 2559-2567.

Plagemann, P.G.W., Marz, R. and Wohlhueter, R.M. (1978). Uridine transport in Novikoff

rat hepatoma cells and other cell lines and its relationship to uridine phosphorylation and phosphorolysis. **J. Cell. Physiol. 97**: 49-72.

Plagemann, P. G. W., and Woffendin, C. (1986). Effect of Ca^{2+} channel antagonists on nucleoside transport in human erythrocytes and cultured cell lines. **Pflugers Arch. 407**: S32.

Plagemann, P.G.W. and Wohlhueter, R.M. (1984). Nucleoside transport in cultured mammalian cells. Multiple forms with different sensitivity to inhibition by nitrobenzylthioinosine or hypoxanthine. **Biochim. Biophys. Acta 773**: 39-52.

Plagemann, P. G. W., and Wohlheuter, R. M. (1980). Permeation of nucleosides, nucleic acid bases and nucleotides in animal cells. **Curr. Top. Membr. Transp. 14**: 225-230

Plagemann, P. G. W., Wohlhueter, R. M., and Woffendin, C. (1988). Nucleoside and nucleobase transport in animal cells. **Biochim. Biophys. Acta, 947**: 405-443.

Plagemann, P. G. W. and Richey, D. P. (1974). Transport of nucleosides, nucleic acid bases, choline and glucose by animal cells in culture. **Biochem. Biophys. Acta 344**: 263-305.

Pohl, J., and Brock, N. (1974). Vergleichende untersuchungen zur hemmung des adenosinabbaus in vitro durch dilazep. **Arzneim. Forsch. 24**: 1901-1905

Randerath, K. (1966). **Thin-layer chromatography**. Academic Press, New York, London. pp 219-234.

Ritch, P. S., Glazer, R. I. (1984). Pyrrolo[2,3-d]pyrimidine nucleosides In: **Developments in cancer chemotherapy** (Glazer, R. I. ed) CRC Press Inc. Florida. pp 2-33.

Robbins, S. L., Cotran, R. S., and Kumar, V. (1984). **Pathologic Basis of Disease**. W. B. Saunders Company, Philadelphia, London, Toronto.

Robins, R. K., and Revankar, G. R. (1985). Purine analogs and related nucleosides and nucleotides as antitumor agents. **Medicinal Research Reviews. 5**: 273-296.

Roos, H. and Pfleger, K. (1972). Kinetics of adenosine uptake by erythrocytes, and the influence of dipyridamole. **Molecular Pharm. 8**: 417-425

Rosenberg, T. and Wilbrandt, W. (1957). Uphill transport induced by counterflow. **J. Gen. Physiol. 41**: 289-296.

Savarese, T. M., Ghoda, L. Y., and Parks, R. E. Jr. (1983). Biochemical considerations in the design of analogs of 5'-deoxy-5'-methylthioadenosine. In: **Development of Target-Oriented Anticancer Drugs** (Cheng, Y.-C., Goz, B., and Minkoff, M., eds) Raven Press, New York, pp. 129-142

Schabel, F. M. (1979). Test systems for evaluating the antitumor activity of nucleoside analogs. In: **Nucleoside analogues. Chemistry, biology and medical applications.** (Walker, R. T., De Clercq, E., and Eckstein, F. eds) Plenum Press, New York and London. pp 363-394.

Schrader, J., Berne, R. M. and Rubio, R. (1972). Uptake and metabolism of adenosine by human erythrocyte ghosts. **Am. J. Physiol. 223**: 159-166.

Schilcher, R. B., Haas, C. D., Samson, M. K., Young, J. D. and Baker, L. H. (1986). Phase I evaluation and clinical pharmacology of tricyclic nucleoside 5'-phosphate using a weekly intravenous regimen. **Cancer Res. 46**: 3147-3151.

Schwenk, M., Hegazy, E. and Lopez del Pino, V. (1984). Uridine uptake by isolated intestinal epithelial cells of guinea pig. **Biochim. Biophys. Acta 805**: 370-374.

Selby, P. J., Thomas, J. M., Monaghan, P., Sloane, J., and Peckham, M. J. (1980). Human tumor xenografts established and serially transplanted in mice immunologically deprived by thymectomy, cytosine arabinoside and whole-body irradiation. **Br. J. Cancer 41**: 52-61.

Sherman, I. W. (1988). Mechanisms of molecular trafficking in malaria. **Parasitology, 96**: S57-S81.

Shi, M. M., Wu, J-S. R., Lee, C-M., and Young, J. D. (1984). Nucleoside transport.Photoaffinity labelling of high-affinity nitrobenzylthioinosine binding sites in rat and guinea pig lung. **Biochem. Biophys. Res. Comm. 118**: 594-600

Silverberg, E., and Lubera, J. A. (1989). Cancer statistics, 1989. **Ca-A Cancer Journal for Clinicians. 39**: 3-20

Slotman, B. J., and Rao, B. R. (1988). Ovarian cancer (review). Etiology, diagnosis, prognosis, surgery, radiotherapy, chemotherapy and endocrine therapy. **Anticancer Research 8**: 417-434

Sobrero, A. F., Moir, R. D., Bertino, J. R., and Handschumacher, R. E. (1985). Defective facilitated diffusion of nucleosides, a primary mechanism of resistance to 5-fluoro-2'-deoxyuridine in the HCT-8 human carcinoma line. **Cancer Res. 45**: 3155-3160.

Sollevi, A., Torssell, L., Owall, A., Edlund, A., and Lagerkranser, M. (1987). Levels and

cardiovascular effects of adenosine in humans. In: **Topics and Perspectives in Adenosine Research** (Gerlach, E., and Becker, B. F., ed) Springer-Verlag, Berlin and Heidelberg. pp 599-613

Spector, R., and Huntoon, C. (1982). Specificity and sodium dependence of the active nucleoside transport system in choroid plexus. **J. Neurochem. 42**: 1048-1052

Steck, T. L. (1974). The organization of proteins in the human red blood cell membrane. A review. **J. Cell Biol. 62**: 1-19.

Stein, W.D. (1986). **Transport and Diffusion Across Cell Membranes**. Academic Press. New York, London.

Suhadolnik, R. J. (1970). **Nucleoside Antibiotics**. Wiley Interscience. New York, London, Sydney, Toronto.

Suhadolnik, R. J. (1979). Pyrrolopyrimidines nucleoside analogs. In: **Nucleosides as Biological Probes** John Wiley Sons, Inc., New York, pp. 158-169.

Taube, R. A., and Berlin, R. D. (1972). Membrane transport of nucleosides in rabbit polymorphonuclear leukocytes. **Biochim. Biophys. Acta. 255**: 6-18.

Thigpen, J. T., Vance, R. B., Balducci, L. and Khansur, T. (1984). New drugs and experimental approaches in ovarian cancer treatment. **Sem. Oncol. 11**: 314-326.

Thigpen, J. T., Vance, R. B., and Lambuth, B. W. (1988). Ovarian carcinoma: The role of chemotherapy. **Sem. Oncol. 15; Supp. 3**: 16-23.

Trias, J., Rosenberg, E. Y. and Nikaido, H. (1988). Specificity of the glucose channel formed by protein D1 of *Pseudomonas aeruginosa*. **Biochim. Biophys. Acta 938**: 493-496.

Turner, J. R. and Moran, A. (1982a). Stoichiometric studies of the renal outer cortical brush border membrane D-glucose transporter. **J. Memb. Biol. 67**: 73-80.

Turner, R. J., and Moran, A. (1982b). Further studies of proximal tubular brush border membrane D-glucose transport heterogeneity. **J. Memb. Biol. 70**: 37-45.

Turnheim, K., Plank, B., and Kolassa, N. (1978). Inhibition of adenosine uptake in human erythrocytes by adenosine-5'-carboxamides, xylosyladenine, dipyridamole, hexobendine, and p-nitrobenzylthioguanosine. **Biochem. Pharmacol. 27**: 2191-2197

Ungemach, F.R. and Hegner, D. (1978). Uptake of thymidine into isolated rat hepatocytes. Evidence for two transport systems. **Hoppe-Seyler's Z. Physiol. Chem. 359**: 845-856.

Van Belle, H. (1970). The disappearance of adenosine in blood. Effect of lidoflazine and other drugs. **Eur. J. Pharmacol. 11**: 241-248

Van Belle, H. (1988). *In vivo* effects of inhibitors of adenosine uptake. In: **Adenosine and Adenine nucleotides: Physiology and Pharmacology** (Paton, D. M. ed.) Taylor & Francis, London, Philadelphia, New York. pp 251-258

Vijayalakshmi, D., and Belt, J. A. (1988). Sodium-dependent nucleoside transport in mouse intestinal epithelial cells. Two transport systems with differing substrate specificities. J. **Biol. Chem. 263**: 19419-19423.

Ward, B. G., Wallace, K., Shepherd, J. H., and Balkwill, F. R. (1987). Intraperitoneal

xenografts of human epithelial ovarian cancer in nude mice. **Cancer Res. 47:** 2662-2667.

Warnick, C. T., Muzik, H., and Paterson, A. R. P. (1972). Interference with nucleoside transport in mouse lymphoma cells proliferating in culture. **Cancer Res. 32:** 2017-2022.

West, I. C. (1983). **The Biochemistry of Membrane Transport.** Chapman and Hall. London, New York.

White, J.C., Hines, L.H. and Rathmell, J.P. (1985). Inhibition of 1-β-D-arabinofuranosylcytosine transport and net accumulation by teniposide and etoposide in Ehrlich ascites cells and human leukemic blasts. **Cancer Res. 45:** 3070-3075.

Widdas, W. F. (1952). Inability of diffusion to account for placental glucose transfer in the sheep and consideration of the kinetics of a possible carrier transfer. **J. Physiol. 118:** 23-29.

Widdas, W. F. (1954). Facilitated transfer of hexoses across the human erythrocyte membrane. **J. Physiol. (Lond) 125:** 163-180.

Widdas, W. F. (1988). Old and new concepts of the membrane transport for glucose in cells. **Biochim. Biophys. Acta, 947:** 385-404.

Wieth, J. O. (1971). Effects of hexoses and anions on the erythritol permeability of human red cells. **J. Physiol. (Lond) 213:** 435-453.

Wilbrandt, W. (1975). Criteria in carrier transport. **Biomembranes 7:** 11-30.

Wilbrandt, W., and Rosenberg, T. (1961). The concept of carrier transport and its corollaries

in pharmacology. **Pharmacol. Rev. 13:** 109-183

Wiley, J. S., Jones, S. P., and Sawyer, W. H. (1983). Cytosine arabinoside transport by human leukaemic cells. **Eur. J. Cancer Clin. Oncol. 19:** 1067-1074.

Woffendin, C., and Plagemann, P. G. W. (1987). Interaction of [^{3}H]dipyridamole with the nucleoside transporters of human erythrocytes and cultured animal cells. **J. Membr. Biol. 98:** 89-100.

Wohlhueter, R. M., Marz, R., Graff, J. C., and Plagemann, P. G. W. (1976). The application of rapid kinetic techniques to the transport of thymidine and 3-O-methylglucose into mammalian cells in suspension culture. **J. Cell Physiol. 89:** 605-612.

Wohlhueter, R.M., Marz, R., Graf, J.C. and Plagemann, P.G.W. (1978a). A rapid-mixing technique to measure transport in suspended animal cells: applications to nucleoside transport in Novikoff rat hepatoma cells. **Meth. Cell Biol. 20:** 221-226.

Wohlhueter, R. M., Marz, R., and Plagemann, P. G. W. (1978b). Properties of the thymidine transport system of chinese hanster ovary cells as probed by nitrobenzylthioinosine. **J. Memb. Biol. 42:** 247-264.

Wohlhueter, R. M., and Plagemann, P. G. W. (1980). The roles of transport and phosphorylation in nutrient uptake in cultured animal cells. **Int. Rev. Cytology, 64:** 171-240

Wohlhueter, R. M., and Plagemann, P. G. W. (1982). On the functional symmetry of nucleoside transport in mammalian cells. **Biochim. Biophys. Acta. 689:** 249-260

Woolf, B., qouted by Haldane, J. B. S., and Stern, K. G. (1932). Allgemeine chemie der enzyme, **Steinkoppf Verlag, Dresden.** pp 119.

Woodcock, D. M. (1987). Cytosine arabinoside toxicity: molecular events, biological consequences, and their implications. **Sem. Oncol. 14; Suppl 1:** 251-256

Wu, J-S. R., Kwong, F. Y. P., Jarvis, S. M., and Young, J. D. (1983). Identification of the erythrocyte nucleoside transporter as a band 4.5 polypeptide.Photoaffinity labelling studies using nitrobenzylthioinosine. *J. Biol. Chem. 258:* 13745-13751

Wu, J-S. R., and Young, J. D. (1984). Photoaffinity labelling of nucleoside-transport proteins in plasma membranes isolated form rat and guinea-pig liver. **Biochem. J. 220:** 1-8

Young, J.D. (1978). Nucleoside transport in sheep erythrocytes: Genetically controlled transport variation and its influence on erythrocyte ATP concentrations. **J. Physiol. 277:** 325-339.

Young, J.D. and Jarvis, S.M. (1983). Nucleoside transport in animal cells. **Biosci. Rep. 3:** 309-322.

Young, J. D., and Jarvis, S. M. (1985). The use of ligands in the study of the nucleoside-transport complex.Nitrobenzylthioinosine. In:**Methods in Pharmacology 6:** 181-190 (Paton, D. M. ed) Plenum Press, New York.

Young, J. D., Jarvis, S. M., Belt, J. A., Gati, W. P., and Paterson, A. R. P. (1984). Identification of the nucleoside transporter in cultured mouse lymphoma cells. **J. Biol. Chem. 259:** 8363-8365

Young, J. D., Jarvis, S. M., Robins, M. J., and Paterson, A. R. P. (1983). Photoaffinity labelling of the human erythrocyte nucleoside transporter by N^6-(p-azidobenzyl)adenosine and nitrobenzylthioinosine. Evidence that the transporter is a band 4.5 polypeptide. J. **Biol. Chem. 258**: 2202-2208

Zimmerman, T. P., Mahony, W. B., and Prus, K. L. (1987). 3'-azido-3'-dideoxythymidine an unusual nucleoside analogue that permeates the membrane of human erythrocytes and lymphocytes by nonfacilitated diffusion. **J. Biol. Chem. 262**: 5748-5754.